

CHARACTERIZATION OF THE RABBIT RENAL H,K-ATPASES

by

W. GRADY CAMPBELL

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1998

This thesis is dedicated to my father, William Maxwell Campbell, and my mother, Clara Hicks Campbell.

ACKNOWLEDGMENTS

I would like to acknowledge the contributions of a number of people who helped me in this work. First I would like to thank my committee, the chair Dr. Brian Cain, Dr. Charles Wingo, Dr. Susan Frost, Dr. Harry Nick, and Dr. Michael Kilberg. Also Dr. David Weiner was very generous to share his lab and microscopes. Dr. Jill Verlander directed a very interesting line of research to answer some questions that arose out of our own research.

I would also like to thank my fellow lab members for putting up with me all this time. Thanks go to Kim McCormick, Philip Hartzog, Abbe Stack, Jim Gordon, James Gardner, Paul Sorgen, Tammy Caviston, and Regina Perry. James, Paul, Tammy, Regina, and I shared many years together as a particularly cohesive lab family. Also I want to thank my labmates in my lab home away from lab home, Jeanette Lynch, Amy Frank, and Robin Moudy. They really went out of their way for me, and it was a pleasure working with them. Mary Handlogten was also especially helpful in getting me started in protein work. I want to acknowledge the contribution of the Yang lab, including Mike Litt, Chien Chen, and Sue Lee. Having an adjacent lab with open doors and shared space between them has turned out to be a great design, there has been a high level of camaraderie and exchange of ideas between the labs.

I also appreciate the support and encouragement of my family, my sister Diane, her children Emory and Lisacole, my brother-in-law Ken, and the newest addition to our family Samma. The companionship and support of Ruth has helped me throughout my time here. And most of all, I appreciate the enthusiastic support of my father and late mother during my years here.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABBREVIATIONS	x
ABSTRACT	xiii
BACKGROUND AND SIGNIFICANCE	1
Overview	1
H,K-ATPase in the Kidney	4
H,K-ATPase Structure and Function	17
Renal Tissue Culture Cells	23
Summary	26
MATERIALS AND METHODS	27
Molecular Biology	27
Biochemistry	36
Fluorescence Microscopy	40
H,K-ATPASE β SUBUNITS IN THE RABBIT RENAL MEDULLARY COLLECTING DUCT	45
Introduction	45
Renal Medulla HK β mRNA Variant	46
Discussion	49
H,K-ATPASE α SUBUNITS IN THE RABBIT RENAL CORTICAL COLLECTING DUCT	52
Introduction	52
Multiple H,K-ATPase α Subunits in the Kidney	53
Alternative Splicing of H,K-ATPase α Subunits in the Kidney	69
Expression of H,K-ATPase α Subunits in the Kidney	72
Discussion	76

H,K-ATPASE ACTIVITY IN A RABBIT KIDNEY CORTICAL	
COLLECTING DUCT CELL LINE	81
Introduction	81
Detection of H,K-ATPase in RCCT-28A Cells	82
Discussion	100
PERSPECTIVE AND FUTURE DIRECTIONS	106
Multiplicity of H,K-ATPase Isoforms in the Kidney	106
Cell Type Specificity of H,K-ATPase in the Kidney	112
Future studies	113
REFERENCES	117
BIOGRAPHICAL SKETCH	129

LIST OF TABLES

<u>Table</u>	<u>page</u>
2-1. PCR primer pairs	32
2-2. Solutions for determination of pH_i	41

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
1-1. Schematic diagram of H,K-ATPase	18
3-1. Northern analysis showing presence of HK β mRNA in renal cortex, renal medulla, and stomach	47
3-2. 3' and 5' RACE reactions to amplify HK β cDNAs using rabbit renal medulla as template	48
3-3. HK β and β' subunit mRNAs	50
4-1. Design of degenerate primers for RT-PCR of novel P-type ATPases	56
4-2. RT-PCR product amplified from rabbit renal cortex RNA using degenerate primers	57
4-3. BLAST search using sequence of 419 bp fragment of HK α_2	58
4-4. Cloning of HK α_{2a} and HK α_{2c} cDNAs	59
4-5. GenBank accession records for rabbit HK α_2 sequences	61
4-6. Northern analysis showing presence of HK α_{2a} in distal colon and renal cortex	67
4-7. Distance analysis of selected HK α and NaK α subunit coding	70
4-8. Rabbit HK α_2 gene sequence at the 5' end	71
4-9. Western analysis showing presence of HK α_{2a} and HK α_{2c} protein in renal cortex	73
4-10. Immunohistochemistry by Dr. Jill Verlander and Ms. Robin Moudy	75
5-1. Southern blots of H,K-ATPase subunit mRNA in RCCT-28A cells	84

5-2. HK β and HK α_1 subunit mRNA in RCCT-28A cells	86
5-3. HK α_2 subunit mRNA in RCCT-28A cells	88
5-4. Western analysis showing presence of HK α_{2c} protein in RCCT-28A cells	90
5-5. pH _i recovery from an acid load by RCCT-28A cells in the absence of Na ⁺	96
5-6. pH _i recovery from an acid load by RCCT-28A cells in the presence of EIPA	98
5-7. Summary of the rates of pH _i recovery from an acid load by RCCT-28A cells	102

ABBREVIATIONS

ATP	adenosine triphosphate
BCECF	2',7'-bis(carboxyethyl)-5(6)- carboxyfluorescein
BCECF-AM	acetoxymethyl ester of BCECF
bp	base pairs
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EIPA	ethylisopropylamiloride
FBS	fetal bovine serum
FITC	fluoroisothiocyanate
HEPES	N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK α	H,K-ATPase α (catalytic) subunit
HK α_1	H,K-ATPase α_1 subunit
HK α_{2a}	H,K-ATPase α_{2a} subunit
HK α_{2b}	H,K-ATPase α_{2b} subunit
HK α_{2c}	H,K-ATPase α_{2c} subunit
HK α_3	H,K-ATPase α_3 subunit

HK α_4	H,K-ATPase α_4 subunit
HK β	H,K-ATPase β subunit
HK β'	H,K-ATPase β' subunit
hr	hour
kb	thousand base pairs
kDa	thousand Daltons
M	molar
mEq	milliequivalents
min	minute
MW	molecular weight
NaK α	Na,K-ATPase α (catalytic) subunit
NaK α_1	Na,K-ATPase α_1 subunit
NaK α_2	Na,K-ATPase α_2 subunit
NaK α_3	Na,K-ATPase α_3 subunit
NaK α_4	Na,K-ATPase α_4 subunit
NaK β_1	Na,K-ATPase β_1 subunit
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH _i	intracellular pH
PMSF	phenylmethylsulfonyl fluoride
pS	picoSiemen
RACE	Rapid Amplification of cDNA Ends

RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
sec	second
Tris	tris[hydroxymethyl]aminomethane
UTR	untranslated region

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF THE RABBIT RENAL H,K-ATPASES

By

W. Grady Campbell

August 1998

Chairperson: Dr. Brian D. Cain

Major Department: Biochemistry and Molecular Biology

H,K-ATPases are located on the apical membrane of epithelial cells lining the collecting duct in the kidney. ATP hydrolysis drives luminal acidification and potassium reabsorption by the enzyme. H,K-ATPases consist of two subunits. The catalytic sites are located on the α subunits, and the β subunits play a role in intracellular trafficking. The goal of this work was to elucidate the molecular identity of H,K-ATPase α and β subunits in kidney. Northern analysis demonstrated two H,K-ATPase β subunit species in rabbit renal medulla; only the smaller of these was present in renal cortex and gastric tissues. A search for H,K-ATPase α subunit isoforms in rabbit renal cortex was conducted using reverse transcriptase-polymerase chain reaction with degenerate primers. A full-length cDNA of the H,K-ATPase α_2 subunit was obtained. Two 5' ends of this transcript were observed by 5' Rapid Amplification of cDNA Ends. One (HK α_{2a}) had high homology to

previously known H,K-ATPases, and the second was a novel variant (α_{2c}). Phylogenetic analysis showed that the rabbit α_{2a} subunit clusters near H,K-ATPases from human axillary skin and rat distal colon, but is more distant from gastric H,K-ATPase and Na,K-ATPases. Gene sequence analysis showed that the first HK α_{2c} exon was located within the first HK α_{2a} intron. Western analysis using anti-peptide polyclonal antibodies demonstrated the expression of HK α_1 , HK α_{2a} and HK α_{2c} subunits in rabbit renal cortex. The mRNAs encoding the HK β , HK α_1 , HK α_{2a} , and HK α_{2c} subunits were detected in the rabbit cortical collecting tubule intercalated cell line RCCT-28A. These results indicated that acid-secreting intercalated collecting duct cells were a cell type containing H,K-ATPase, and these cells appear capable of expressing multiple H,K-ATPase isoforms.

BACKGROUND AND SIGNIFICANCE

Overview

H,K-ATPase was first identified in the stomach, where it is expressed at an extremely high level to mediate the extrusion of acid that aids digestion. A great deal has been learned about regulation of H,K-ATPase in the stomach, enough to feel as though its regulation is understood in a meaningful way. At the molecular level, gastric-specific transcription factors have been found that may be responsible for the marked tissue-specific regulation of expression of H,K-ATPase (Maeda *et al.*, 1991; Oshiman *et al.*, 1991; Tamura *et al.*, 1992). The principal means of induction of gastric H,K-ATPase from its basal state is by unwinding a tightly coiled tubular network, exposing pumps localized in the network and thereby increasing the size of the secretory surface (Pettit *et al.*, 1995). This morphological transformation is triggered by various stimuli including activation of stretch receptors in the stomach by entering food, the sight of food, or even the thought of food (reviewed by Wolfe and Soll, 1988; Hersey and Sachs, 1995). Thus, there is a framework of understanding of the regulation of H,K-ATPase in the stomach at the molecular and cellular levels, and the root stimuli that result in the H,K-ATPase response are known.

H,K-ATPase is expressed at much lower level in the kidney, but its role in the kidney is even more critical to life than its role in the stomach. The kidney plays the major role in

K⁺ homeostasis, maintaining extracellular (including plasma) K⁺ concentration within the relatively narrow normal range of 3.8 to 5.0 mEq/L (Merck, 1992; Guyton and Hall, 1997) despite a wide variation in K⁺ intake. If plasma K⁺ levels depart significantly from these values, then cardiac arrhythmias lead to life-threatening conditions.

K⁺ transport in the kidney is also important to blood pressure regulation. It has been observed that urinary K⁺ levels and urinary Na⁺/K⁺ ratio are related to systolic and diastolic blood pressures (INTERSALT, 1988; Whelton, 1993). Excretion rates show a higher degree of correlation with elevated blood pressure than serum levels, implicating the kidney as an important organ in regulating the effect of K⁺ on blood pressure. Dietary K⁺ supplementation has been shown to lower blood pressure since early this century (Ambard and Beaujard, 1904; Addison, 1928).

The substantial role of renal ion transport in blood pressure regulation is emphasized by studies conducted by Lifton (1996), who determined the molecular bases underlying certain types of monogenic inherited extreme hypo- and hypertension disorders. Each of the genetic defects found affected renal ion transport. Such well-defined inherited diseases of blood pressure regulation are not common. In 95% of people experiencing hypertension their diagnosis is essential hypertension, the root cause being essentially unknown. To understand this majority of hypertensive disorders, a more complete understanding of renal ion transport is needed. Because of the role of H,K-ATPase in the final regulation of K⁺ excretion in the kidney, understanding its contribution to renal ion transport could well be important in understanding the problem of essential hypertension.

In order to understand the role of H,K-ATPase in the kidney, the exact H,K-ATPase pumps of the kidney must be characterized at a molecular level. This characterization must

be carried out to a high level of detail, to explore variations in the ion pumps conferred by differences in changes in transcriptional start or adenylation sites, or by alternative splicing. With the identity of the H,K-ATPase subunits known at a molecular level, various reagents can then be developed to characterize H,K-ATPase regulation. These include cDNA probes, antibodies, and activity assays. With these tools available, it will be possible to develop a more complete framework of understanding of renal H,K-ATPase.

The studies in this dissertation contribute to our knowledge of the H,K-ATPase subunits at a molecular level. At the time these studies began, it was not known what H,K-ATPase subunits were responsible for the active H^+ and K^+ exchange that had been observed in renal collecting duct. Further evidence is presented that all the currently known H,K-ATPase subunits are present in kidney, and that the primary cell type in which all are expressed is the collecting duct acid-secreting intercalated cell. When these studies were begun, there was no known alternative splicing of P-type ATPases. Here alternative splicing of an $HK\alpha$ subunit mRNA is described, and expression of a protein product is shown in kidney. In addition, a variant renal medulla $HK\beta$ transcript is described that has tissue-specific expression even within the kidney. In summary, this work has generated a new appreciation for the complexity of H,K-ATPase molecules that underlie renal collecting duct H,K-ATPase activity.

H,K-ATPase in the Kidney

Transport activity

The kidney is the principal organ responsible for potassium homeostasis and plays a major role in maintenance of the acid-base balance of the body. The renal collecting duct (CD) is the primary site of regulation of the excretion of potassium and the acidification of urine. A number of studies implicate an apical H,K-ATPase as an important mediator of these functions. The enzyme (pump) actively transports H^+ into the lumen of the nephron in a nonelectrogenic exchange for K^+ (for review, see Wingo and Cain, 1993, Wingo and Smolka, 1995).

A K^+ activated ATPase was observed in frog gastric microsomes by Ganster and Forte (1973). Lee *et al.* (1974) found that gastric microsomes isolated from dog mucosa were able to accumulate H^+ in the presence of ATP and K^+ . These studies were the initial observations of the now well-known gastric H,K-ATPase. The designation of H,K-ATPase was given the enzyme by Sachs *et al.* (1976).

Gustin and Goodman (1981) isolated apical brush-border membrane of the rabbit descending colon by isolation of epithelial cells, homogenization, and centrifugation on a Percoll gradient. They found a membrane-bound, K^+ -activated ATPase, which had a $K_{act}=2 \times 10^{-4}M$ for K^+ , was competitively inhibited by Na^+ , but had no activation by Na^+ . It was vanadate sensitive, but oligomycin and ouabain (1mM) insensitive. This represented the first observations of enzymatic activity for the colonic H,K-ATPase.

Smolka and Sachs (in Sachs *et al.*, 1982), employing monoclonal antibodies, detected a protein at least similar to the gastric proton-potassium-translocating ATPase protein in

renal distal tubule and colon. Sachs *et al.* (1982) advanced the idea that this protein might be involved in the K^+ reabsorption or the acidification known to take place in kidney or colon.

By quantitating the hydrolysis of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, Doucet and Marsy (1987) observed a K^+ -stimulated ATPase activity in rabbit kidney. The level of activity was related to the density of intercalated cells in microdissected segments of rabbit connecting segment (highest activity), cortical collecting duct (intermediate), and outer medullary collecting duct (lowest), and not detectable in any other nephron segments. The ATPase affinity for K^+ was high ($K_m=0.2\text{-}0.4\text{mM}$). The pharmacological properties of the renal H,K-ATPase was examined as a preliminary indication of the pumps present in the kidney. Omeprazole is an inhibitor of the gastric isoform of H,K-ATPase, vanadate inhibits all P-type ATPases, and ouabain is a Na,K-ATPase inhibitor that also inhibits non-gastric H,K-ATPase isoforms. The renal ATPase was inhibited by omeprazole and vanadate, but not by ouabain. They also observed a K^+ -ATPase activity with potassium restriction in rat renal outer medullary collecting duct that roughly doubled, changing little after 0.5 weeks low- K^+ diet. In cortical collecting duct activity doubled, rising steadily during a five week low- K^+ diet (Doucet and Marsy, 1987).

Wingo (1987) did a more complete study of dietary K^+ influence on K^+ transport in the kidney. He first established that rabbits on a K^+ -replete diet, or on a K^+ -depleted diet, or on a K^+ -deplete Na^+ -supplemented diet all consumed similar quantities. Wingo (1987) used restricted diets containing 0.55% K^+ , marginally less than the 0.6% generally thought to be required for normal rabbit growth. In time-course studies he found that after an initial two-week period of K^+ -replete meals, 72 hr was sufficient for the response to

equilibrate as judged by urinary Na^+ and K^+ excretion. Muscular K^+ levels had not altered significantly in that time, showing that renal response preceded effects deleterious to the animal. Serum K^+ levels were slightly higher in K^+ -replete animals, and the same in K^+ -depleted Na^+ -supplemented animals compared to K^+ -depleted animals. The sodium supplementation evidently maintained the K^+ serum level. Serum aldosterone was found to be 2.88 ± 0.57 ng/dl in K^+ -depleted Na^+ -supplemented rabbits, 15.6 ± 5.3 for K^+ -depleted rabbits, and 44.7 ± 14.0 for potassium-replete animals, and thus any changes in transport observed cannot be correlated with aldosterone level.

In his experiments, Wingo (1987) found that perfused collecting ducts from outer medullary inner stripe had similar rates of fluid reabsorption, gauged by ^3H -inulin flux, and similar transepithelial voltages. K^+ reabsorption, however, roughly tripled among rabbits on K^+ -restricted diets, measured as a flux of ^{42}K . K^+ -replete rabbits had $5.9 \pm 0.8\%$, K^+ -depleted rabbits had $17.2 \pm 2.0\%$, and K^+ -depleted Na^+ -supplemented rabbits had $20.8 \pm 4.2\%$ reabsorption. The data were not significantly different in either of the two K^+ -restricted cases. He concluded that K^+ reabsorption in the medullary collecting tubule is comparable to K^+ secretion in the cortical segments at normal fluid flux rates, and could have important contributions to the amount of K^+ excreted in urine. In a later report by Wingo (1989), the name H,K-ATPase was first explicitly given to the K^+ -stimulated ATPase that had been studied by others in the kidney.

Utilizing a fluorometric microassay in which ATP hydrolysis is coupled to the oxidation of NADH, Garg and Narang (1989) detected the presence of a K^+ -dependent, ouabain-insensitive ATPase activity in rabbit kidney that was also omeprazole-, SCH28080-, and vanadate-sensitive. Sch-28080, like omeprazole, is a potent gastric

H,K-ATPase inhibitor. Earlier work by this group (Garg and Narang, 1988) noted H⁺ secretion coupled to an ATPase that was not NEM-inhibited, and could not account for the vacuolar H⁺-ATPase they were studying. Significant K⁺-ATPase activity was seen in microdissected connecting segment (17.0 ± 3.3 pmol min⁻¹ mm⁻¹), cortical collecting duct (6.6 ± 0.7), and outer medullary collecting duct (8.8 ± 1.7), but not in proximal straight tubule, convoluted tubules, nor the thick ascending limbs. They found the K⁺-ATPase activity to be affected by diet. Activity was found in the connecting segment (13.0 ± 4.0 pmol min⁻¹ mm⁻¹), cortical collecting duct (10.1 ± 3.0), and outer medullary collecting duct (10.8 ± 2.2) in animals on a low K⁺ diet. Cortical collecting duct activity varied with pH; optimal pH of 7.4 gave activity of 10 pmol min⁻¹ mm⁻¹, falling at lower pH to 3 at pH of 7.0, and falling at higher pH to 5 at pH 7.8. Thus, a high K⁺ diet completely suppressed the K⁺-ATPase activity, while rabbits fed a normal diet had similar activity to the low K⁺ fed rabbits of the second study.

Studies by Cheval and co-workers (Cheval *et al.*, 1991) examined the ⁸⁶Rb flux (⁸⁶Rb is a K⁺ analog used as a radioactive tracer for transporter studies) and K-ATPase activities. These activities were blocked by Sch-28080 in cortical and medullary collecting duct. Activities they attributed to Na,K-ATPase and H-ATPase were unaffected by Sch-28080. In this study, ouabain was used at a concentration of 2.5 mM to inhibit Na,K-ATPase. Although there are inconsistencies in the exact concentration at which ouabain inhibits H,K-ATPases containing the HKa₂ subunit, all expression studies carried out to date (Modyanov *et al.*, 1995; Codina *et al.*, 1996; Cougnon *et al.*, 1996; Grishin *et al.*, 1996) except for one (Lee *et al.*, 1995) indicate that 2.5 mM ouabain is sufficient to block the HKa₂ enzyme. Lee *et al.* (1995) detected no ouabain sensitivity at 1 mM, and it is not

known what effect 2.5 mM would have had in their system. In this work by Cheval *et al.* (1991) the activities of Na,K-ATPase and an HK α_2 H,K-ATPase would be indistinguishable.

These early studies do not distinguish between different isoforms of H,K-ATPase that may together account for the H,K-ATPase activity observed in the kidney. Given historical perspective, this makes sense because at the time these studies were conducted, only HK α_1 and HK β subunit isoforms were known, and the ouabain-sensitive H,K-ATPase was not yet known. Later studies (Younes-Ibrahim *et al.*, 1995; Buffin-Meyer *et al.*, 1997) addressed the issue of multiple isoforms of H,K-ATPase in the kidney and their distribution along the nephron and collecting duct. These investigators found three distinct H,K-ATPase activities. Two were found in the collecting duct; one of these was sensitive to Sch-28080; the other was insensitive. The third was found in proximal tubules and thick ascending limbs, and was inhibited by Sch-28080. In normal rats, the sole H,K-ATPase activity present in collecting duct was the Sch-28080-sensitive activity. In K⁺-depleted rats, the overall H,K-ATPase activity in collecting duct increased, while the activity in proximal tubule decreased. The increase was abolished by ouabain, but not by Sch-28080, implying that the increase is due to an H,K-ATPase isoform that is pharmacologically dissimilar to HK α_1 . The proximal tubule/thick ascending limb H,K-ATPase may have a basolateral polarity, rather than the apical localization of the collecting duct H,K-ATPase. If the H,K-ATPase role in proximal tubule and thick ascending limb involves K⁺ homeostasis, then its down regulation by dietary K⁺ depletion would imply a basolateral location. Together, these results argue for three different H,K-ATPase isoforms in kidney. One was constitutively expressed in the collecting duct,

and was Sch-28080-sensitive and ouabain-insensitive, like the $\text{HK}\alpha_1$ isoform of H,K-ATPase . The second was presumably located basolaterally in proximal tubule and thick ascending limb, reduced by low K^+ , and was ouabain-sensitive. The third was located apically in the collecting duct, stimulated by low K^+ , and Sch-28080-insensitive but ouabain-sensitive.

Earlier studies naturally concentrated on establishing the existence of H,K-ATPase in the kidney. The early studies demonstrated H,K-ATPase activity in connecting segment, cortical collecting duct, and outer medullary collecting duct. Although measurements were made for H,K-ATPase activity in other nephron segments, no H,K-ATPase activity was found outside the connecting segment and collecting duct. More recently, proximal tubule and thick ascending limb have been added as regions having H,K-ATPase activity. These studies also began to address the differences among H,K-ATPases that are present in the kidney, defining the characteristics of the H,K-ATPase activities and their localization. The identity of the H,K-ATPase molecules responsible for the two collecting duct activities have been found. The identity of the H,K-ATPase molecule responsible for the activity in the more proximal nephron is not yet known.

H,K-ATPase subunit isoforms in the kidney

Until the last five years, the presence of H,K-ATPase in the kidney has been defined only on the basis of activity. We have now begun to determine the molecular identities of the pumps responsible for this activity. And only very recently it has become appreciated that the complexity of H,K-ATPase expression also includes alternative splicing of $\text{HK}\alpha$ and $\text{HK}\beta$ isoforms.

An H,K-ATPase has long been known to acidify the lumen of the stomach. This pump is a member of the P-type ATPase family, which shares similarity of sequence, structure and mechanism. The gastric H,K-ATPase is comprised of a catalytic α_1 subunit and a β subunit; the active form of the enzyme is an $(\alpha\beta)_2$ oligomer (for review see Hershey and Sachs, 1995; Van Driel and Callaghan, 1995). Full-length cDNAs for rabbit HK α_1 (Bamberg *et al.*, 1992) and HK β (Reuben *et al.*, 1990) have been cloned and sequenced. RT-PCR followed by sequencing of the amplified products has been used to demonstrate that mRNA (Ahn and Kone, 1995) and protein (Callaghan *et al.*, 1995) for HK α_1 and that mRNA and protein for HK β (Callaghan *et al.*, 1995) isoforms are present in kidney. This author was included in the latter study.

The non-gastric H,K-ATPase α subunit isoforms (HK α_2) have been cloned from several tissues. A partial cDNA for an HK α_2 isoform was obtained from human axilla skin, and mRNA observed in brain and kidney (Modyanov *et al.*, 1991). A full-length cDNA was found subsequently (Grishin *et al.*, 1994). An HK α_2 cDNA was cloned from rat distal colon by Crowson and Shull (1992); the corresponding amino acid sequence has 86% amino acid identity to the cDNA derived from human skin. This rat HK α_2 mRNA was detected in kidney, uterus, and heart using two separate cDNA probes from HK α_2 3' UTR and C-terminal transmembrane domains. The 3' UTR derived probe detected HK α_2 in forestomach as well. These human and rat cDNAs were shown to encode H⁺/K⁺ exchange activity when expressed in *Xenopus laevis* oocytes (Modyanov *et al.*, 1995; Cougnon *et al.*, 1996), although recent work questions the stoichiometry of the exchange in the human isoform (Grishin *et al.*, 1996). In two of these studies rabbit HK β subunits were

cotransfected (Modyanov *et al.*, 1995; Grishin *et al.*, 1996), and in the other *Bufo marinus* HK β subunits were cotransfected (Cougnon *et al.*, 1996). A partial HK α_2 cDNA was cloned from a rabbit cortical CD (CCD) library having 84% amino acid identity to the human HK α_2 , and mRNA was detected in CCD and colon (Fejes-Toth *et al.*, 1995). Watanabe *et al.* (1992) cloned and sequenced a similar cDNA from distal colon of guinea pig. The degree of identity at the amino acid level among human, rat, guinea pig, and rabbit HK α_2 clones is less than the amino acid identity of HK α_1 (>97%) between the three species, but much greater than the typical amino acid identity between HK α_1 and other P-type ATPases (<64%). Therefore, although controversy on this point exists, this author considers these to be orthologous and refers to them collectively as HK α_2 . As part of this dissertation, further evidence will be presented that the HK α_2 genes are indeed orthologs. A related cDNA (75% identity at the amino acid level) was cloned from *Bufo marinus* bladder, an analog of mammalian collecting duct (Jaisser *et al.*, 1993). While mRNA was detected in toad bladder, none was observed in either stomach or colon. The evolutionary distance between toad and mammal coupled with the tissue distribution dissimilarity of the toad HK α make it difficult to evaluate the relationship between the toad and mammalian isoforms. In addition to uncertainty in the number of H,K-ATPase isoforms in the kidney, it has been recently been discovered that there are two alternatively spliced transcripts of the rat HK α_2 in kidney (Kone, 1996; Higham and Kone, 1998). Thus, several H,K-ATPase α subunit isoforms have been reported in kidney, but it is at present uncertain whether these account for all the renal H,K-ATPases. Also uncertain is their relative contributions to renal H,K-ATPase activity.

Experiments have been conducted in attempts to find more members of the gene family that includes the α subunits of Na,K-ATPases and H,K-ATPases. Shull and Lingrel (1987) probed a human genomic library at low stringency with probes made from Na,K-ATPase sheep α_1 , rat α_1 , rat α_2 , and rat α_3 . Five different sequences were obtained, with two to ten clones representing each sequence. Three of these were known to be human NaK α_1 , NaK α_2 , and HK α_1 genes. The fourth was later identified as HK α_2 (Modyanov *et al.*, 1991). The fifth, which is physically linked to the NaK α_2 , was later identified as NaK α_4 by Shamraj and Lingrel (1994). In a similar experiment, Sverdlov *et al.*, (1987) screened a human genomic library with a probe made from porcine kidney NaK α_1 . The probe was constructed to contain the well-conserved region surrounding the active site aspartate residue. They obtained five distinct clones. They were recognized as the three known NaK α subunits and the two known HK α subunits. In sum, the results of these two experiments imply that all members of the gene family of X,K-ATPases are known.

At present, there are no known specific HK β isoforms in addition to the one originally discovered in gastric tissues. However, it is thought that the NaK β_1 subunit isoform is the partner to HK α_2 in active H,K-ATPase pumps in colon and kidney (DuBose *et al.*, 1998; Kraut *et al.*, 1998). These observations are the first suggesting that an individual P-type ATPase β subunit has more than one primary P-type ATPase α subunit partner *in vivo*. This was the determination reached by two separate groups using different antibodies to specifically immunoprecipitate $\alpha\beta$ pairs, lending strength to their independent and identical conclusions. However, it is possible that during tissue processing some of the α/β pairs

may mix partners, leading to an erroneous conclusion. Pairs that are not thought to associate *in vivo* have been seen in expression systems to give rise to functional activity (Horisberger *et al.*, 1991; Codina *et al.*, 1996), showing that the *in vivo* P-type ATPase pairs are not exclusive when expressed in expression systems. Expressed in their proper cell types, there may be compartmentation that controls HK α and HK β selectivity. This could segregate the subunits from their incorrect partners on the basis of translation of the various H,K-ATPase and Na,K-ATPase isoforms at different times or in different locations.

Alternative transcriptional start sites for HK β in the stomach were detected by Newman and Shull (1991). The primer extension method used by Newman and Shull (1991) would not have necessarily differentiated between alternative transcriptional start sites and alternative splicing. Primer extension would only give information about the distance from the primer to the beginning of any or all of the transcripts that contain the primer site. So it is possible that they were detecting alternative splicing as well as alternate transcription start sites. Thus far, however, there have been no reports of multiple HK β subunit transcripts in any tissue seen by RNA analysis techniques such as northern analysis or ribonuclease protection assay. In present work, I present evidence that the transcriptional start site of HK β subunit in the medulla has slight differences relative to stomach, and that in renal medulla there are two transcripts expressed at comparable level, one of which appears to be the product of alternative splicing.

In summary, the first H,K-ATPase subunit detected in kidney was the non-gastric isoform HK α_2 , shown by RT-PCR to be present in human kidney (Modyanov *et al.*, 1991). Shortly before this author joined his lab, Dr. Brian Cain found that the rabbit β

subunit isoform of stomach was present in kidney (Callaghan *et al.*, 1995; Campbell-Thompson *et al.*, 1995). After the work described here was begun, work by other investigators (Ahn and Kone, 1995) established that the catalytic subunit isoform found in stomach, HK α_1 , is also found in kidney. HK α_1 protein was shown by immunoblot in rabbit kidney (Callaghan *et al.*, 1995). To further complicate the picture, an alternatively spliced HK α_2 (dubbed HK α_{2b} , with the canonical HK α_2 being renamed HK α_{2a}) was discovered in kidney (Kone and Higham, 1998). A search for the β subunit partner to HK α_{2a} established that NaK β_1 couples to HK α_{2a} *in vivo* (Dubose *et al.*, 1998; Kraut *et al.*, 1998). Presumably, HK α_1 pairs with HK β in kidney as it does in stomach (Hall *et al.*, 1991; Shin and Sachs, 1994, Mathews *et al.*; 1995). All the known H,K-ATPase subunit isoforms are known in kidney. HK α_1 /HK β and HK α_2 /NaK β_1 H,K-ATPases contribute to the activities observed in the collecting duct. Experiments have been done to find other candidate H,K-ATPase genes, and to date none have been detected. So far no molecular identity can be associated with the H,K-ATPase activity in the proximal tubule and thick ascending limb.

Deficiencies of H,K-ATPase

There are two lines of evidence to indicate that H,K-ATPases are critical to maintenance of K⁺ homeostasis, and that disturbances in this balance are a serious health problem. First, transgenic mice with deranged H,K-ATPase function show substantial disturbances of K⁺ balance (Meneton *et al.*, 1998). Second, an environmentally high level

of vanadium in northeast Thailand effectively inhibits H,K-ATPase function among humans and water buffalo living in the area (Dafnis *et al.*, 1993).

Experiments have been carried out to show the critical nature of H,K-ATPase expression and its relevance in the kidney (Meneton *et al.*, 1998). Meneton showed that transgenic mice homozygous with respect to an HK α_2 subunit deletion were normal when fed diets that had normal levels of K⁺ (1% K⁺). However, when fed K⁺-free diets (<0.004% K⁺) these mice experienced loss in body weight, plasma K⁺, and muscle K⁺. HK α_2 is not the major mechanism of K⁺ conservation in the kidney; the urinary K⁺ excretion rate in both wild-type and HK α_2 -deficient mice declined 100-fold compared to HK α_2 -deficient mice on normal diets. However, mean urinary K⁺ excretion per day was consistently higher (typically 120% of wild-type) following a week of K⁺-free feeding. This suggests that there may be a role played by renal HK α_2 in K⁺ conservation. The role played by HK α_2 in the colon was more clear. Fecal excretion rate increased four-fold in HK α_2 -deficient compared to normal mice that had been fed the same K⁺-free diet, indicating an inability to reabsorb K⁺ by the digestive tract. Cardiac arrhythmias were observed in some of the HK α_2 -deficient mice, presumably due to low plasma K⁺.

Anecdotal evidence concerning H,K-ATPase activity in humans comes to us from Thailand, where environmental vanadium levels are high. Vanadate is a transition state inhibitor for all P-type ATPases, and its presence in drinking water and soil in northeast Thailand was associated with an epidemic of renal distal tubular acidosis. It is interesting that although vanadate would be expected to inhibit Na,K-ATPases and Ca-ATPases, along with H,K-ATPase in stomach and distal colon, the predominant symptoms were

renal. The disease is characterized by an inability to lower urine pH to below 5.5 pH units. Affected persons had generalized paralysis, hypokalemia, metabolic acidosis, muscle and bone pain, and nocturia (Nilwarangkar *et al.*, 1990). The connection between acid secretion in the kidney and stomach had been made, and some patients tested positive for gastric hypoacidity (Sitprijia *et al.*, 1988). However, the exact cause of the acid secretion defect was unknown. As recently as 1990, a genetic predisposition had not been ruled out (Nilwarangkar *et al.*, 1990). Patients are treated by K^+ and alkali supplements, and some deaths occurred with non-compliance.

It was shown in rats that intraperitoneal injections of vanadate (5 mg/kg) led to hypokalemic distal renal tubular acidosis and loss in muscle K^+ (Dafnis *et al.*, 1993). Cortical collecting duct K-ATPase activity that was sensitive to Sch-28080 (200 μ M) declined 75% in vanadate-treated rats. Medullary collecting duct activity declined less than 50% by the same assay. However, it should be noted that this is a sufficiently high Sch-28080 concentration to inhibit $HK\alpha_2$ enzyme activity according to some investigators (Modyanov *et al.*, 1995; Grishin *et al.*, 1996), but other studies have not seen sensitivity to Sch-28080 even at that relatively high concentration (Cougnon *et al.*, 1996; Codina *et al.*, 1996). Therefore, a vanadate effect on $HK\alpha_2$ enzyme activity in this study might have remained undetected. Na,K-ATPase activity was measured also, and showed a decline in vanadate-treated animals as well. This assay also may or may not have detected a contribution due to $HK\alpha_2$ activity, which is expected to be ouabain-sensitive at concentrations of between $>10 \mu$ M (Modyanov *et al.*, 1995) and <1 mM (Codina *et al.*, 1996) depending on the study. The evidence suggests that vanadate could be a factor in the endemic hypokalemic distal renal tubular acidosis in northeast Thailand. More precise

measurements need to be made to quantitate the effect of Sch-28080 and ouabain on $\text{HK}\alpha_2$ activity.

In summary, these studies illustrate the essential nature of H,K-ATPases in the renal collecting duct (and in distal colon) for the maintenance of K^+ balance. In the extreme case, deranged H,K-ATPase function can result in disease or even death. These studies isolating the activity of H,K-ATPase do not address the role H,K-ATPase might play in blood pressure regulation. The other more acute affects of impairments of H,K-ATPase activity probably obscured the affects on blood pressure regulation. Although in some cases a defect in a single gene leads to a dramatic loss in blood pressure control, hypertension is most often a multifactorial disease. H,K-ATPase is likely to be one of a combination of activities that fail to control blood pressure in the hypertensive patient.

H,K-ATPase Structure and Function

H,K-ATPase catalytic subunit

The $\text{HK}\alpha$ subunit contains the active sites relating to its catalytic action (Figure 1-1). A number of functionally important sites in the $\text{HK}\alpha$ have been defined. The location of the phosphorylated aspartyl residue of the catalytic intermediate is known and the region immediately surrounding it is extremely well conserved within the P-type ATPase family (Walderhaug *et al.*, 1985). The location of a residue that binds the fluorescent molecule FITC is also known; FITC competes with ATP for binding to H,K-ATPase and therefore is thought to be at or near the $\text{HK}\alpha$ ATP binding site (Jackson *et al.*, 1983; Farley and Faller, 1985). The site of the K^+ -competitive inhibitor Sch-28080 which acts at an

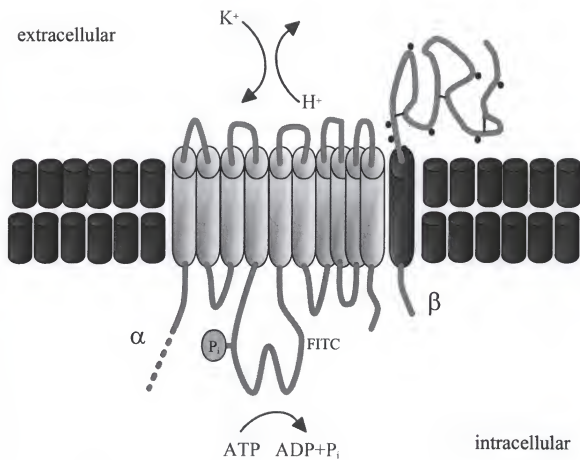


Figure 1-1. Schematic diagram of H,K-ATPase. Dashed line indicates the amino-terminal extension of HK α_{2c} protein.

extracellular site (Munson and Sachs, 1988) is known by studies with a photoaffinity analog to lie between the first two transmembrane domains (Munson *et al.*, 1991). The Na,K-ATPase inhibitor ouabain binds to the corresponding site in that enzyme, but it is not known whether this is the binding site conferring ouabain's less sensitive inhibition of HK α_2 . The binding site of the medically important inhibitor omeprazole is also known, it may bind to any of three extracellular cysteines located in the C-terminal quarter of the subunit (Besancon *et al.*, 1993). This inhibitor has achieved some popular acclaim, television advertisements may be seen for it under its brand name Prilosec.

Using hydropathy analysis, the existence of four transmembrane helices in the amino-terminal half of HK α_1 is clear. The number of transmembrane domains of the carboxyl-terminal half of HK α is not as apparent from the hydropathy plot, but may be placed somewhere between three and five by that analysis. However, the presence of an odd number of membrane-spanning regions would be inconsistent with the placement of amino- and carboxy-termini in the cytoplasm by antibody reactivity (Smolka *et al.*, 1992; Mercier *et al.*, 1993). Also, the localization of the active sites discussed in the preceding paragraph, predicts a large intracellular cytosolic loop following the first four transmembrane domains. The picture is further complicated because recent results (Raussens *et al.*, 1998) imply that some of the transmembrane domains may be comprised of β -strand secondary structure. Using limited tryptic digestion followed by fluorescent labelling of cysteines, the four transmembrane segments of the amino-terminal half could be confirmed (Besancon *et al.*, 1993; Shin *et al.*, 1993). In the carboxyl-terminal half of the protein, only three transmembrane domains were observed. The two most carboxyl-terminal membrane spans strongly implied by hydropathy analysis were not

observed by fluorescent labelling despite the presence of multiple cysteines. *In vitro* translation experiments have also been performed to elucidate H,K-ATPase topology (Bamberg and Sachs, 1994). These experiments involve synthesis of putative transmembrane segments in the presence and absence of microsomes, then detection of glycosylation of the resulting polypeptides by electrophoresis. Positive glycosylation of a fusion protein containing glycosylation sites would imply the insertion of a single transmembrane domain. When a pair of membrane spanning domains were translated, no glycosylation would result. Both single transmembrane domains and pairs of transmembranes were tested. In this system, the predicted final two transmembrane segments were inserted into the membrane. Taken together these experiments predict a secondary structure for HK α_1 that has amino- and carboxy-termini located intracellularly. There are four transmembrane domains in the amino-terminal half of the protein, and between four and six transmembrane domains in the carboxyl-terminal half. If H,K-ATPase membrane topology is conserved with that of Ca-ATPase, recently imaged by cryoelectron microscopy (Zhang *et al.*, 1998), then HK α_1 subunits probably have ten membrane spans.

The significance of H,K-ATPase enzyme quaternary structure is presently under active consideration. Studies suggest that there are specific and stable associations between catalytic subunits. Radiation inactivation studies showed the minimum functional unit to be an ($\alpha\beta$)₂ heterotetramer (Rabon *et al.*, 1988). The electron microscope diffraction pattern in images of two-dimensional H,K-ATPase crystals suggested a tetrameric arrangement of HK α_1 subunits (Hebert *et al.*, 1992). The apparent contradiction of the radiation inactivation and electron microscopy experiments can be explained. An α/α interaction has

been shown to be necessary for optimal activity of H,K-ATPase (Morii *et al.*, 1996). Dimeric α/α interactions were necessary for H,K-ATPase activity, and tetrameric α/α interactions confer higher affinity binding of ATP. When coexpressed in insect cells, NaK α_1 , NaK α_2 , and NaK α_3 were found to coimmunoprecipitate (Blanco *et al.*, 1994). Using chimeric constructions combining NaK α_1 and HK α_1 alternating regions, it was found that the large intracellular loop was necessary for this association (Koster *et al.*, 1995). Using a yeast two-hybrid system and fusions of Gal4 to α subunit cytosolic loops Colonna *et al.* (1997) further explored this interaction. In two-hybrid assays, there was no apparent direct interaction between pairs of the large cytosolic loop (Figure 1-1). Likewise there was no interaction observed between the smaller loop between the second and third transmembrane domains. However, the two-hybrid assay gave a positive interaction between the smaller and larger loops. The interaction is apparently between the loop between transmembrane domains two and three of one member of the α/α pair and the larger loop between transmembrane domains four and five of the other. These results imply that interactions between H,K-ATPase molecules play a role in their function. There may be interactions with other molecules as well, such as those governing cell polarity.

H,K-ATPase β subunit

Antibodies to the HK β subunit protein (Chow and Forte, 1993) and reduction of any of the three disulfide bonds in the HK β subunit (Chow *et al.*, 1992) have been observed to affect catalytic subunit function. In addition to a role in modulating enzymatic activity, the HK β subunit is also thought to participate in shepherding the H,K-ATPase through the

Golgi and to the plasma membrane (Renaud *et al.*, 1991). The HK β subunit contains a tyrosine-based signal required for internalization of the H,K-ATPase pump (Courtois-Coutry *et al.*, 1997) and thus required for proper regulation of pump activity. The HK β subunit protein contains 7 consensus N-glycosylation sites and all are glycosylated (Chow and Forte, 1993). Thus, the HK β subunit is required for H,K-ATPase pumps reaching the cell surface and proper function of the enzyme. Its role in internalization means that it is required to down-regulate gastric acid secretion.

Hydropathy analysis predicts a single membrane domain for the HK β subunit protein. Interactions between HK α and HK β subunit proteins have been studied by limited tryptic digestion. After digestion, detergent solubilization, and lectin binding of the HK β subunit, an HK α subunit fragment corresponding to the putative loop between transmembrane domains seven and eight was recovered (Shin and Sachs, 1994). The sequence of this fragment is well-conserved, implying some important function. Yeast two-hybrid analysis has confirmed this area of the HK α subunit protein (*Arg-898* to *Arg-922*) as a region of association with the HK β subunit (Melle-Milovanovic *et al.*, 1998). The yeast two-hybrid analysis has also shown that two extracellular domains of the HK β protein to be regions of association with the HK α subunit. The HK β subunit amino acids involved are *Gln-64* through *Asn-130* (adjacent to the membrane) and *Ala-156* to *Arg-188*.

The HK β subunit is quite important to H,K-ATPase activity due to its role in intracellular trafficking of the holoenzyme. It apparently also affects the conformation of the intact complex, because HK β -specific effects modify enzymatic activity. Just as there are α/α interactions that are now known, the regions of the subunit important to α/β

interactions are also becoming known. These may be important in conferring the effects on enzyme activity conferred by oligomeric structure. They may also be important in controlling the interaction between the various α and β subunit proteins of the H,K-ATPases and Na, K-ATPases.

Renal Tissue Culture Cells

A number of renal continuous cell lines exist, but for the purpose of studying H,K-ATPase relevance to the cortical collecting duct the selection narrows considerably. In cortical collecting duct H,K-ATPase activity is relatively high, and based on immunohistochemical (Wingo *et al.*, 1990) and *in situ* hybridization evidence (Campbell-Thompson *et al.*, 1995; Ahn and Kone, 1995). H,K-ATPase is found in intercalated cells in cortical collecting duct. There are two cell lines with characteristics of these cell types, MDCK cells and RCCT-28A cell.

The Madin-Darby canine kidney (MDCK) cell line is an epithelial cell line that appears to have originated from distal tubule (Herzlinger *et al.*, 1982) or cortical collecting tubule (Valentich, 1981), and has properties of intercalated cells (Pfaller *et al.*, 1989). MDCK cells are aldosterone-responsive (Simmons, 1978), and immunostaining of H^+/K^+ ATPase has been observed (Adam Smolka, personal communication). Oberleithner *et al.* (1990) detected an aldosterone-stimulated, omeprazole-inhibited transport activity that was blocked by increased apical extracellular $[H^+]$, or decreased apical extracellular $[K^+]$, consistent with the presence of an apical H^+/K^+ ATPase in MDCK cells. MDCK cells are available for purchase from the American Type Culture Collection (ATCC, Rockville, MD). Because of the uncertainty of their origin, and because we wanted to take full

advantage of the knowledge of rabbit renal physiology accumulated over the years by our collaborator Dr. Charles Wingo, we selected the RCCT-28A cell line for use in our experiments.

The RCCT-28A transformed cell line was derived from immunodissected rabbit CCD by Arend *et al.* 1989. The RCCT-28A line was created by microdissecting cortical collecting tubule and dispersing cells on plates coated with monoclonal antibody specific to collecting tubule cells. The antibody coating the plates, known as IgG₃(rct-30), had been made against rabbit renal cortical cells injected into mice. The resultant monoclonal antibody stained only collecting tubules on cryotome sections. Staining was primarily basolateral in intercalated and principal cells. Cells from the dissected rabbit collecting duct that had bound in the antibody-coated dish were immortalized with an adenovirus 2-SV40 hybrid, and then cloned by limited diffusion. A population of cells that continued to proliferate while retaining epithelial morphology was obtained.

The antigenic and hormone response of these cells is specifically consistent with their origin in the cortical collecting tubule. Immunocytochemistry showed reactivity in 100% of cells to an antibody (mr-mct) against mitochondria-rich cells of the medullary collecting duct (Schweibert *et al.*, 1992). The mr-mct antibody was seen to be specific for acid-secreting, or type A cells (Burnatowska-Hledin and Spielman, 1988). Immunoreactivity was also observed to an antibody to band 3 protein (IVF12), another marker for acid-secreting intercalated cells (Schweibert *et al.*, 1992). The presence of carbonic anhydrase in >95% of cells was indicated by the binding of a fluorescent acetazolamide analog (Dietl *et al.*, 1992). As expected for an intercalated cell, conductance of Cl⁻, but not Na⁺ or K⁺, was indicated by patch clamp measurements (Dietl

et al., 1992). Schweibert *et al.* (1992) saw no antigenicity of the cells toward two antibodies specific for base-secreting (or type B) cells or toward four antibodies specific for principal cells. The origin, collection, and characterization of these cells indicates that they are a good model of the acid-secreting intercalated collecting duct cell.

The first study undertaken using these cells showed that adenosine analogs increase intracellular calcium by stimulating phosphoinositide turnover (Arend *et al.*, 1989). Inositol turnover was measured by labeling cells with myo-[^3H]inositol and detecting [^3H]inositol phosphate formation. A1-receptor agonists increased phosphoinositide turnover, and the increase was blocked by an A1-receptor antagonist. Adenosine also regulated a 305 pS chloride channel in RCCT-28A cells via protein kinase C and a G protein (Schwiebert *et al.*, 1992). Chloride channels in these cells have been characterized by the patch clamp method, showing Cl^- conductance to be stimulated by isoproterenol (Dietl *et al.*, 1992; Dietl and Stanton, 1992). Cell swelling of RCCT-28A cells activated a Cl^- conductance by altering the organization of actin filaments (Mills *et al.*, 1994; Schwiebert *et al.*, 1994). Activation of the channel was mimicked by stretching the membrane and disruption of F-actin by dihydrocytochalasins. Stabilizing F-actin with phalloidin blocked activation of the Cl^- channel. Bello-Reuss (1993) reported H,K-ATPase activity in RCCT-28A cells, observing an apical acidification mechanism that had a component sensitive to withdrawal of K^+ and two H,K-ATPase inhibitors, Sch-28080 and omeprazole. She also detected activities suggestive of an apical H-ATPase and a basolateral Cl/HCO_3^- exchanger. Acid secretion by these cells was diminished in cells grown at low pCO_2 , evidence of regulation of the acidification mechanism by alkaline conditions. These cells have proven useful in studying various processes normally

associated with acid-secreting intercalated cells. The observation of H,K-ATPase activity in these cells made them particularly appealing for our studies.

Summary

H,K-ATPase activity in the collecting duct has very real implications for maintenance of health and well-being. Studies have shown a myriad of derangements in the absence of functional H,K-ATPase activity, including a real possibility of death. The H,K-ATPase provides an excellent example of how critical some of the enzymes are that fine tune the environment of the body. It is interesting that an enzyme that has such an immense level of activity in one organ, the stomach, actually is more important in another, the kidney, in which its level of activity might be called small.

Several challenges exist in studying the H,K-ATPase enzyme and its function. One of these is the lack of knowledge of the quantitative effects of ouabain and Sch-28080 on the HK α_2 isoform of the enzyme. Another its low level of expression, making assays of mRNA, protein, and activity difficult. There was also a dearth of knowledge about the molecular forms of H,K-ATPase in the kidney when these studies were undertaken. This situation has changed, and the contributions described herein have been part of that change.

MATERIALS AND METHODS

Molecular Biology

Tissue culture

The RCCT-28A cell line was derived from immunodissected renal cortical collecting duct (Arend *et al.*, 1989). These cells were the kind gift of Dr. William Spielman, and experiments were performed using cells between passages 11 and 31. Cells were grown in DMEM media supplemented to 10% with FBS and to 1% with Penicillin-Streptomycin. All media was bubbled overnight with a mixture of 5% CO₂, 21% O₂, 74% N₂, then filter sterilized by use of a 0.20 µm cellulose acetate syringe-mounted filter (Corning, Corning, NY). Cells were maintained in culture in tissue culture flasks at 37°C in a 5% CO₂ atmosphere. Media was changed on alternate days, and cells were split 4:1 at confluency. For experiments, cells were passaged to Corning Costar Transwell Collagen-coated semipermeable inserts. Cells were plated to a density of $2 \times 10^5/\text{cm}^2$ on the inserts, grown for 2 days in media containing 10% FBS, and then shifted to 0.1% FBS for a period of 24 hr prior to the experiment.

Isolation of total RNA

Total RNA was isolated from cells or tissues employing the method described by Chomczynski and Sacchi (1987). After aspiration of media, tissue culture cells were rinsed in ice cold sterile PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl). Next, 0.5 mL of GTC solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% n-lauroylsarcosine, 100 mM β -mercaptoethanol) was added to promote cell lysis. The resulting viscous fluid was scraped from the insert and emptied into polypropylene centrifuge tubes on ice. New Zealand White Rabbits were sacrificed by decapitation and the kidneys removed immediately. Kidneys were dissected under a microscope, slicing coronally to separate cortex and medulla. Distal colon was prepared by clipping the distal 25 mm of colon, cutting longitudinally, then rinsing away contents by a thorough spray of ice cold PBS. Gastric mucosa was collected by slicing the stomach transversely, rinsing in ice cold PBS, then scraping the rugae with a Scoopula (Fisher, Pittsburgh, PA). Tissues were dounce homogenized in 4 mL GTC solution until the suspension appeared to be homogeneous. The resulting viscous fluid was poured into polypropylene centrifuge tubes on ice.

While being held on ice, 1 volume phenol was added to the homogenized tissue, then 1/10 volume 2 M sodium acetate (pH 4.0), and 0.22 volume chloroform-isoamyl mixture (24:1). After each addition, the samples were briefly vortexed. The samples were subjected to centrifugation at 1000G X 25 min at 4°C. The upper phase was retained, and RNA was precipitated twice in isopropanol and twice in ethanol, then resuspended in 100 μ L DEPC-treated water. Storage of the RNA was at -20°C. RNA concentration was

determined by OD₂₆₀ reading on a spectrophotometer. For absorbance of 1.0 at 260 nm, the concentration was taken to be 40 µg/ml.

Northern analysis

Northern blots were done following the procedures of Sambrook *et al.* (1989). Samples of total RNA (20 µg per lane) or mRNA (2 µg per lane) underwent electrophoresis in a 1% agarose, 0.22 M formaldehyde denaturing gel. Capillary transfer to a nylon membrane (Hybond N, Amersham Corp., Arlington Heights, IL) was conducted overnight in 20X SSC (3M NaCl, 300 mM Na citrate). Absorbent paper towels were changed twice during transfer. RNA is immobilized on the membrane by baking 2 h at 80°C in a vacuum oven. ³²P-labelled probes were prepared by random primer extension of 75-150 ng DNA according to the protocol of the Megaprime Kit (Amersham). Membranes were prehybridized a minimum of 15 min and hybridized with labelled probe for 24 h in hybridization solution at 65°C. When heterologous probes were employed, such as cross-species probing, temperatures as low as 42°C were used to reduce stringency. Washing was done first at room temperature for 20 min in 1X SSC, 0.1% SDS, then 3 times at hybridization temperature for 20 min in 0.2X SSC, 0.1% SDS. After washing, membranes were exposed to Kodak BioMax MS film at -80°C with Kodak BioMax intensifying screens. Exposures between three and six days were often required to detect H,K-ATPase subunit mRNA. An mRNA probe for glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme, was used as a control to ensure even loading amounts of RNA between lanes. An exposure of eight hr was typically sufficient to visualize this control.

RT-PCR using degenerate primers

RT-PCR was carried out as described by Davis *et al.*, 1994. The cDNA template for the PCR reaction was produced by incubating 1 µg total RNA from microdissected rabbit renal cortex and 0.1 µg random hexamers in a volume of 11 µL at 70°C X 2 min. The reaction mixture was quenched by placing the tube on ice. The reverse transcription reactions were carried out at 37°C X 1 hr in a volume of 25 µL. The mixture contained RNA-random hexamers mix in the final concentrations indicated: First Strand Buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂), dithiothreitol (10mM), dNTPs (2mM), RNasin (30 U, Promega, Madison, WI), and Superscript II (200 U, Gibco BRL, Gaithersburg, MD). PCR reactions were primed with pairs of oligonucleotides shown in Table 2-1 synthesized by the University of Florida Interdisciplinary Center for Biotechnology Research (UF ICBR) DNA Synthesis Core. PCR reactions were performed in a volume of 100 µL at the final concentrations indicated: dNTPs (0.22 mM), PCR Buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), MgCl₂ (50 mM), primers (1 µg each), and *Taq* DNA Polymerase (5 U). The reactions were overlaid with 50 µL mineral oil. Thermal parameters of the reactions included a 5 min X 94°C presoak followed by 94°C X 40 sec denaturation, 55°C X 1 min anneal, and 72°C X 2 min extension for a total of 30 cycles followed by a final extension of 5 min. Reactions were held at 4°C overnight before PCR product cloning into the pCR II vector and transformed into OneShot *E. coli* cells utilizing the TA Cloning Kit (Invitrogen, San Diego, CA). *E. coli* cells containing the plasmid of interest were grown overnight in a 5 mL culture and plasmids isolated using the

QiaPrep Spin Mini Kit (Qiagen, Santa Clarita, CA). Sequencing of plasmids was carried out by the UF ICBR DNA Sequencing Core.

RT-PCR using standard primers

A reverse transcription reaction was carried out as in the RT-PCR method described above for degenerate primers. PCR reactions were primed by pairs of oligonucleotides synthesized by the UF ICBR DNA DNA Synthesis Core and summarized in Table 2-1. Reactions were carried out using 1 µg total RNA in a 50 µL volume in KlenTaq PCR Reaction Buffer (40 mM Tricine-KOH, pH 9.2, 15 mM KOAc, 3.5 mM Mg(OAc)₂, and 75 µg/mL Bovine Serum Albumin), primers as listed above (0.5 µM), and dNTPs (0.8 mM), and Advantage KlenTaq Polymerase Mix (Clontech, Palo Alto, CA). Thermal parameters of the reactions included a 2 min X 94°C presoak followed by 94°C X 30 sec denaturation and 68°C X 1 min extension with a final extension of 5 min. The numbers of cycles were specific to each experiment and are indicated in the legends. PCR reactions were purified for sequencing by phenol/chloroform (1:1) extraction and two 3000 G X 5 min centrifugation steps in Ultrafree-MC 30000 NMWL regenerated cellulose columns (Millipore, Bedford, MA). Due to low yield of the HKα1 PCR reaction, a 45 cycle reaction was carried out to generate product for sequencing. Sequencing of PCR products was carried out by the UF ICBR DNA Sequencing Core.

Genomic PCR

To amplify genomic DNA for sequencing, template used was 1 µg Clontech Rabbit Genomic DNA. Clontech KlenTaq Advantage PCR Mix was used with components and

Table 2-1. PCR primer pairs

Isoform Nucleotides	Sense Primer	Serial #	Antisense Primer	Serial #
HK α_1 2537-3147	CTGCACCGACATTTTCCCGTCCG	BC204	TGCTGGGCAACAGCGAACCCA	BC203
HK α_{2a} 7-93	TGCCCCGCCGACCCGCGGCCTCCA	BC381	TATCTGTAGCTGCATGGTGCTCCAC	BC334
HK α_{2a} 56-337	AGARATTTAYTCYGTRGARTC	BC328	TTGATGATCTCTGGGGTCTG	BC 265
HK α_{2a} 285-1033	MGGGAYGGNCCTAAYRCNCT	BC198	AAGATGATGGCGTCCAGGAC	BC250
HK α_{2a} 974-1569	CATCATTTTCTTCATCAYCGC	BC243	GCTTGTCATTGGGATCTTCC	BC231
HK α_{2a} 1182-1600	ATCTGCTCNGACAAACNGG	BC199	TCNGGGGCNCCYTTTCATCAC	BC200
HK α_{2a} 1264-1569	CCGACACGAGTGAAGACAAT	BC230	GCTTGTCATTGGGATCTTCC	BC231
HK α_{2a} 1550-3121	GGAAGATCCCAATGACAAAGC	BC257	TCCCACCAAGCTNCCWGGYA	BC197
HK α_{2c} 24-377	CGGAGAAAAGTGCTAGACTGGAGCT	BC366	AGAGTGCAATACCGGCCCCAGGTGG	BC344
HK β 304-873	TAAGGCCAGACGTGTATGGG	BC92	TGTCGAAGGTCACGTGATCG	BC91

Note: Notation is that of the International Union of Biochemistry Nomenclature Committee: N=any base, R=A or G, M=A or C, W=A or T, and Y=C or T.

concentrations specified in the description of RT-PCR above. Primers were ACCCGCGGCGCCTCCAGCGGACAT (nucleotides 16-40, BC386) located in the first exon of HK α_{2a} and TATCTGTAGCTGCATGGTGCTCCAC (nucleotides 69-93, BC334) located in the second exon of HK α_{2a} . Thermal parameters of the reactions included a 1.5 min X 94°C presoak followed by 5 cycles of 94°C X 15 sec denaturation and 72°C X 2 min extension, 5 cycles of 94°C X 15 sec denaturation and 70°C X 2 min extension, and 25 cycles of 94°C X 15 sec denaturation and 68°C X 2 min extension with a final extension of 8 min. Reactions were held at 15°C overnight before ligation of the products into the pCR 2.1 vector. The ligation mixture was transformed into OneShot *E. coli* cells utilizing the TOPO-TA Cloning Kit (Invitrogen, San Diego, CA). *E. coli* cells containing the plasmid of interest were grown overnight in a 5 mL culture and plasmids isolated using the QiaPrep Spin Mini Kit. Sequencing of plasmids was carried out by the UF ICBR DNA Sequencing Core.

3' RACE

A 3' RACE reaction was carried out as described by Davis *et al.*, 1994. mRNA was prepared from rabbit renal cortex total RNA using the PolyATtract (Promega, Madison, WI) system. A reverse transcription reaction was carried out as in the RT-PCR method described above, using 1 μ g mRNA as template and 0.1 μ g primer (GACTCGAGTCGACATCGA[T]₁₇, BC229). The complementary strand was next synthesized using 5 μ L of RT reaction along with 0.1 μ g of sense primer (TGCGGAAACTCTTCATCAGG, nucleotides 3088-3107, BC262) in a reaction volume of 98 μ L that contained the following components: PCR Buffer (20 mM Tris-HCl, pH

8.4, 50 mM KCl), $MgCl_2$ (50 mM), dNTPs (0.22 mM), overlaid with 50 μ L mineral oil. After a 95°C incubation for 5 min, the temperature was lowered to 70°C and 5 U *Taq* DNA Polymerase was added. A 2 min annealing phase followed at 55°C, then the complementary strand was extended at 72°C for 10 min. Antisense primer (0.1 μ g, GACTCGAGTCGACATCG, BC230) was added, and a PCR reaction was initiated with a 94°C X 40 sec denaturation, followed by a 55°C X 1 min anneal, and a 72°C X 2 min extension for a total of 40 cycles. Final extension was for a duration of 5 min. Reactions were held at 4°C overnight before cloning PCR products into the pCR II vector utilizing the TA Cloning Kit (Invitrogen, San Diego, CA) per manufacturer instructions. The library of clones produced in this manner was screened using an oligo (CTCTACCCTGGCAGCTGGTG, nucleotides 3108-3127, BC261) 5' labeled using the ECL kit (Amersham, Arlington Heights, IL). Sequencing was carried out by the UF ICBR DNA sequencing core.

5' RACE

A 5' RACE reaction was performed using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) following manufacturer's instructions with the following modifications. RT reactions were carried out using 5.5 μ g total RNA of rabbit renal cortex, incubated with the gene-specific primer TTGCCATCTCGCCCCCTCTT (nucleotides 121-102, BC331) for 30 min at 50°C, then at 55°C for 15 min. PCR reactions were carried out using the anchor primer (CCATCCTAATACGACTCACTATAGGGC, AP1) included in the Marathon kit paired with gene-specific primer TATCTGTAGCTGCATGGTGCTCCAC (nucleotides 93-69, BC334). Concentrations

and components of the reactions were detailed above for use of Clontech KlenTaq Advantage Polymerase Mix. Thermal parameters of the reactions included a 1 min X 94°C presoak followed by 5 cycles of 94°C X 15 sec denaturation and 72°C X 1 min extension, 5 cycles of 94°C X 15 sec denaturation and 70°C X 1 min extension, and 25 cycles of 94°C X 15 sec denaturation and 68°C X 1 min extension with a final extension of 8 min.

Evaluation of PCR products

PCR products were analyzed by agarose gel electrophoresis. 20 µL of reactions were run along with 1 mL DNA loading dye (50% glycerol, 1% xylene cyanol, 1% bromophenol blue) on a 1.2% agarose gel in TAE buffer (Tris mM, acetic acid mM, EDTA mM). Low DNA Mass Ladder (Gibco BRL) was electrophoresed alongside PCR products to quantitate DNA concentration, and 100 bp ladder (Gibco BRL) to evaluate the size of the products.

In some cases, products were not visible after ethidium bromide staining, so Southern blotting was used to visualize the products. Procedures followed were as described by Davis *et al.*, (1994). After running products on gels as described above, gels were soaked 30 min in denaturation solution (1.5 M NaCl, .5 M NaOH), and 30 min in neutralization buffer (1 M ammonium acetate, .02 M NaOH). Capillary transfer in neutralization solution and baking 80° X 1 hr was utilized to adhere DNA to nylon membrane (Hybond N). Probes to hybridize specifically to the expected products (Table 2-1) were created using RT-PCR with rabbit renal cortex as template, cloning the inserts using the TA Cloning Kit as described above. Inserts were sequenced to confirm their identity. Probes were labelled using the ECL Direct System(Amersham) following manufacturers instructions. Exposure

to Hyperfilm ECL (Amersham) for a period of two hr was required to visualize the results of PCR reactions.

Biochemistry

Preparation of membrane protein from tissue culture cells

Two clusters of six wells each were used for each experimental condition; this typically yielded 50 μ g of protein. All solutions and glass douncers are cooled on ice, and centrifuges are either refrigerated or located in cold rooms. Cells are held on ice at all times. Cells were rinsed in PBS containing 0.5 mM PMSF, 1.5 mL in top well of Transwell, 2.5 mL in bottom well. Following aspiration of the rinse solution, to each single well was added 0.5 mL PBS, 0.5 mM PMSF, 1 mM EDTA. A cell scraper was used to dislodge cells from the insert. This solution containing cells was pipetted from one well to the next dislodging and gathering all the cells from one cluster. This was repeated with a fresh 0.5 mL solution on the same cluster to remove any remaining cells. The two passes combined for a total of 1 mL, and the other clusters were processed in the same manner.

Cells were spun in a centrifuge for 5 min at 500 X g to remove cellular debris. Supernatant was discarded, and the pellet was resuspended in swelling buffer (tris 10 mM pH 7.8, 1 mM EDTA, 1 mM PMSF, 2 μ M aprotinin, 2 μ M leupeptin, 2 μ M pepstatin) for 15 min. Cells were homogenized by 50 strokes in a glass douncer. After moving cells back to a microfuge tube, 0.11 mL of 10X salts (300 mM NaCl, 20 mM MgCl₂, 10 mM Tris pH 7.8) was added before vortexing. Mixture was spun in a centrifuge for 1 minute at 1000 X g to remove nuclei. Supernatant was retained and spun in a centrifuge for 5 min at

1500 X g. Supernatant was retained and spun in a centrifuge for 30 min at 23000 X g. Cells were resuspended in 20 μ L resuspension buffer (1 volume swelling buffer, 1/10 volume 10X salts) and stored at -20°C .

Preparation of membrane protein from rabbit tissues

Kidney, distal colon, and stomach tissues were obtained in the same manner as for RNA isolation. Care was taken to maintain solutions and apparatus ice cold. Two rabbit distal colons were required per preparation to yield useful concentrations of protein. Tissues were homogenized for 15 seconds at 12500 rpm (setting 8 on Omni-Sorvall tissue homogenizer) in Buffer A (50 mM sucrose, 10 mM Tris pH 7.4, 1 mM EDTA, .1 mM PMSF). After allowing 15 seconds for settling, homogenization was repeated. Three volumes buffer B (250 mM sucrose, 10 mM Tris pH 7.4, 1 mM EDTA, .1 mM PMSF) were added, then homogenate spun in a centrifuge for 10 min at 1000 X g. The supernatant was subjected to centrifugation for 20 min at 10000 X g three times. Final centrifugation was for 1 hr at 100000 X g. After discarding supernatant, pellet was resuspended in 500 μ L loading solution (1 mM Tris, 10 mM MgCl_2 , 150 mM NaCl). After transferring resuspended pellet to glass douncer, dounce is dropped into the douncer, turned three times, then raised and dropped again. This was done ten times. The resultant preparation was stored at -20°C .

Antibodies

Peptides used as immunogens were designed for maximum antigenicity and minimum homology to other proteins. Avoiding homology to other P-type ATPases was particularly

important. Rabbit H,K-ATPase catalytic subunit sequences were scanned using the computer program PEPTIDESTRUCTURE (Genetics Computer Group, 1997) to find regions relatively high in charged, hydrophilic residues (Jameson and Wolf, 1988). Such regions were then searched by BLAST (Altschul *et al.*, 1990) to eliminate those that could be predicted to cross-react with other proteins. Once these constraints were met the candidate sequences were examined using the program MOTIFS (Genetics Computer Group, 1997; Bairoch and Apweiler, 1996) to ensure that there were no potential sites for protein modification that might affect reactivity. In the case of HK α_1 and HK α_{2a} , there was only one region that satisfactorily met all of these criteria. In the case of HK α_{2c} , there were two, one being the region in common with HK α_{2a} , the other in the extended amino terminal region of HK α_{2c} not contained in HK α_{2a} .

Peptides were synthesized with an N-terminal cysteine and conjugated to keyhole limpet hemocyanin (UF ICBR Protein Core) using the Pierce (Rockford, IL) Inject system. Three peptides were used as immunogens, the first was designed to react with a portion of HK α_1 , the second was designed to recognize a portion of HK α_2 found in both HK α_{2a} and HK α_{2c} , the third to a portion unique to HK α_{2c} . The peptide chosen for HK α_1 corresponded to amino acids 569-582 (CLYLSEKDYPGGYAF). The peptide chosen within the common region contained amino acids 18-37 in HK α_{2a} and 79-88 in HK α_{2c} (CDIKKKEGRDGKKDNDLELKR). The peptide chosen within the HK α_{2c} -specific region corresponded to amino acids 13-25 (CGEERKEGGGRWRA). Antipeptide antibodies were raised in chickens by Lofstrand Laboratories (Bethesda, MD). Chickens received boost inoculations at 21 day intervals, and were exsanguinated at day 73 to

produce antisera. Preimmune sera was collected prior to initial inoculation. Yolks of eggs collected over the two week period prior to final bleed were pooled, and immunoglobulins purified from yolk material by the Promega EGGstract method. Concentration of the EGGstracted yolks was determined by the modified Lowry procedure of Markwell *et al.* (1978) and the concentrations adjusted to 2 mg/mL by addition of IgY buffer solution (Promega). Purity and concentration of the IgY obtained was confirmed by non-reducing SDS-PAGE and staining with Coomassie blue.

Western analysis

Protein concentrations in tissue and cell samples were determined by modified Lowry (Markwell *et al.*, 1978). Proteins (10 µg/lane) were separated on 4-20% reducing SDS-polyacrylamide gels (BioRad, Hercules, CA), 10 µg per lane. Vesicle preparations were suspended in buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 3% SDS) and incubated 2 min X 90°C prior to electrophoresis. Gels were rinsed 10 min in TBS (10 mM Tris- HCl pH 7.2, 150 mM NaCl), and 10 min in transfer buffer (20 mM Tris-HCL, 150 mM glycine, 20% methanol, pH 8.3). Proteins were electrotransferred at 104 V, .25 A, 4°C to Hybond ECL nitrocellulose membranes (Amersham) in transfer buffer. Blocking of membranes was done in TBS-T (TBS, 0.1% TWEEN-20) containing sodium azide and 5% non-fat dry milk at 4°C overnight or room temperature for 1 hr. Antibody incubations were one hour each, carried out in TBS-T containing 5% non-fat dry milk. Following blocking and following primary and secondary antibody incubations, immunoblots were rinsed in TBS-T with continuous agitation. This was done three times for one minute each, then twice for 5 min each. Primary antibodies

purified from egg yolk were used at a dilution of 1:200, the anti-chicken IgY-horseradish peroxidase conjugated secondary antibody (Promega) was diluted to 1:10000. When antisera or preimmune sera were used as primary antibodies, dilution was 1:2000. A final wash was carried out in TBS for 10 min, and then antibody reactivity was detected using chemiluminescence (Pierce). Apparent molecular masses were established using the High Mass Range Molecular Weight Markers (BioRad).

Fluorescence Microscopy

Measurement of pH_i

The fluorescent, pH-sensitive dye BCECF-AM was used to directly measure pH_i in RCCT-28A tissue culture cells. BCECF-AM was stored as a stock at -20°C in a 30 mM solution in DMSO. Cells were incubated at room temperature for a period of 30 min in *solution 1* (Table 2-2) with BCECF-AM at a final concentration of 5 μ M. A minimum of 5 min perfusion with *solution 1* delivered at 37°C was allowed to rinse BCECF away at the beginning of each experiment.

Cells were imaged by epifluorescence at 530 nm emission on an inverted microscope (Boyarsky *et al.*, 1988, Weiner and Hamm, 1989) using excitation wavelengths of 440 nm and 490 nm. These wavelengths correspond to the isosbestic point and to a highly pH-sensitive wavelength of BCECF, respectively. The ratio of emission intensities at the two wavelengths is directly proportional to pH over the pH range being studied, and is constant with respect to such variables as cell-to-cell variations in dye uptake and leakage. A field containing approximately 50 cells could be visualized using a Nikon X40, 0.55

Table 2-2. Solutions for determination of pH_i

	Solutions						
	1	2	3	4	5	6	7
NaCl	119.2				102.2	122.2	99.2
Choline Cl		102.2	122.2	119.2			
Ammonia Cl		20			20		20
KCl	3			3			3
KH ₂ PO ₄	2			2			2
Phosphoric acid		2	2		2	2	
Na-acetate	1				1	1	1
Acetic acid		1	1	1			
CaCl ₂	1.2	1.2	1.2	1.2	1.2	1.2	1.2
MgSO ₄	1	1	1	1	1	1	1
Alanine	5	5	5	5	5	5	5
Glucose	8.3	8.3	8.3	8.3	8.3	8.3	8.3
HEPES	25	25	25	25	25	25	25

Note: Concentration units are in mM. Osmolality was adjusted to 290 ± 5 mosmol/kg H₂O by addition of the principal salt. pH was adjusted to 7.40 ± 0.05 by addition of tetramethylammonium-OH. Solutions were bubbled with 100% O₂.

LWD lens on a Nikon Diaphot-TMD inverted microscope. Excitation light was provided by a 100 W mercury lamp. The light was split into two beams by a 470 nm low-pass dichroic mirror. The two split light beams then passed through filters to yield beams of the desired wavelengths. The transmitted light path contained a 440 nm filter; the reflected light path contained a 490 nm filter. Computer-actuated shutters on each light path alternated the incident light wavelengths and minimized the time the cells were subjected to the high intensity light. The two light beams were recombined by a second 470 nm low-pass dichroic mirror. Light was directed to the microscope stage by a 510 nm high-pass mirror, and the emitted light was directed to a Videoscope KS-1381 image intensifier coupled to a Dage 72 CCD camera. Because the optics necessary to image cells grown on inserts required relatively intense incident light, measurement frequency was limited to avoid phototoxicity. Therefore, measurements were made at 30 second intervals. Images were digitized and stored by computer allowing subsequent analysis of single cells using the Image 1/FL software package (Universal Imaging Corp., Westchester, PA).

During measurements, cells were constantly perfused at a rate of ~ 10 mL/min by HEPES-buffered solutions that were continuously bubbled by 100% O_2 and heated to be delivered at a temperature of $37^\circ C$. Switches for the various solutions were located physically near the input to the cell chamber to minimize the time required to switch solutions, and fluid was continuously removed from the opposite side of the chamber by vacuum suction. For some experiments, the apical side of the cells was perfused by different solutions than the basolateral side of the cells by utilizing separate input tubes in the upper and lower chambers of the Transwell inserts.

Cells were acid-loaded using the NH_4Cl prepulse technique. Briefly, cells were incubated with 20 mM NH_4Cl (equimolar substitution for NaCl) for 5 min, then ammonium chloride was removed from the perfusing solution. Addition of inhibitors or K^+ removal began at the start of the ammonium pre-pulse. The ethylisopropylamiloride (EIPA) stock solution was 1 mM in dimethyl sulfoxide (DMSO), and Sch-28080 was 10 mM in DMSO. EIPA was obtained from Research Biochemicals, International (Natick, MA) and Sch-28080 was the kind gift of Dr. James Kaminski at Schering Corporation (Bloomfield, NJ). Inhibitors were stored as stock solutions at -20°C and diluted into the solutions indicated immediately preceding their use.

Calibration of the pH_i measured by BCECF fluorescence was carried out by the nigericin calibration technique of Thomas *et al.* (1979). Calibration solutions (120 mM KCl, 1.2 mM CaCl_2 , 1 mM MgCl_2 , 25 mM HEPES, 14 μM nigericin) were adjusted to 6.6, 6.8, 7.0, 7.2, and 7.4 pH units using 1 M NaOH and HCl. Cells were incubated with each solution for a minimum of three min, and three fluorescence ratio measurements were taken at each pH.

Statistical methods

pH_i recovery rates for individual cells were calculated using least-squares linear regression. Rates were calculated for the period beginning two min after NH_4Cl withdrawal, allowing time for cells to equilibrate after acidification, and ending with Na^+ addition or EIPA withdrawal. Data were collected for independent experiments involving separate passages of cells. pH_i recovery rates for each experimental condition were presented as the mean of the rates determined for the individual experiments $\pm$ SE. Cells

without Na^+/H^+ exchanger activity, defined as an increase in pH, recovery with EIPA removal, were classified as non-viable and excluded from further analysis. $P < 0.05$ by Student's *t*-test was taken as significant.

H,K-ATPASE β SUBUNITS IN THE RABBIT RENAL MEDULLARY COLLECTING DUCT

Introduction

There are multiple H,K-ATPase subunit isoforms that are involved in coupled H^+ for K^+ active exchange in the kidney. These now include the $NaK\beta_1$ subunit that is the newly recognized partner for the $HK\alpha_{2a}$ subunit, in addition to its long-known role as partner to $NaK\alpha_1$. In addition to the complexity involved in having multiple H,K-ATPase isoforms present, there is the possibility of variant transcripts of each H,K-ATPase subunit. Differences in polyadenylation sites, transcription start sites, and alternative splicing in a tissue-specific manner might further complicate the picture.

When rat $HK\beta$ subunit was originally sequenced, multiple transcriptional start sites were observed by primer extension experiments (Newman and Shull, 1991). It was not known what role these various transcriptional start sites play. When the Cain, Wingo and Nick laboratories did preliminary studies concerning the regulation by K^+ status of the $HK\beta$ subunit in rabbits, it was noted that on some northern blots of renal tissues $HK\beta$ subunit mRNA appeared as a doublet. This was evidence for a second H,K-ATPase subunit transcript present at a level comparable to the primary transcript.

Here we show that there is a variant $HK\beta$ subunit mRNA that is expressed in renal medulla, and not in renal cortex or stomach. We have designated the variant mRNA

HK β '. The HK β ' transcript is found in medulla at a level comparable to the quantity of the HK β subunit, and the translation start site is unchanged, so both HK β and HK β ' encode the same protein. HK β ' may be the product of alternative splicing.

Renal Medulla HK β mRNA Variant

In order to investigate the doublet band in northern blots probed with Hk β cDNA probes, RNA was prepared from stomach, renal cortex, and stomach. In northern analyses (Figure 3-1) involving twenty different rabbits in experiments spaced over several years, only single transcripts were observed in either gastric or renal cortical tissues. Gastric and renal cortical transcripts appeared to be of identical size. However, in renal medullary tissues, a doublet was universally visible. Northern analysis showed that the smaller of the two medullary HK β transcripts was the same size as in the other two tissues, and the second renal medullary transcript was larger. The quantity of the second mRNA, HK β ', was generally comparable to the quantity of HK β mRNA.

In order to determine the molecular nature of the different transcripts of the medulla, 3' and 5' RACE experiments were conducted using mRNA isolated from rabbit renal medulla. The rabbit RNA was selected from those that showed prominent HK β subunit upper bands by northern analysis because these would have a higher abundance of the novel transcript. Representative PCR products generated by the 3' and 5' RACE experiment are shown in Figure 3-2. PCR of ten 3' RACE products sequenced, all were the same, and all extended to within 5 bp of the published HK β cDNA sequence (Reuben *et al.*, 1990). Ten 5' RACE products were also sequenced. Nine of these were similar to

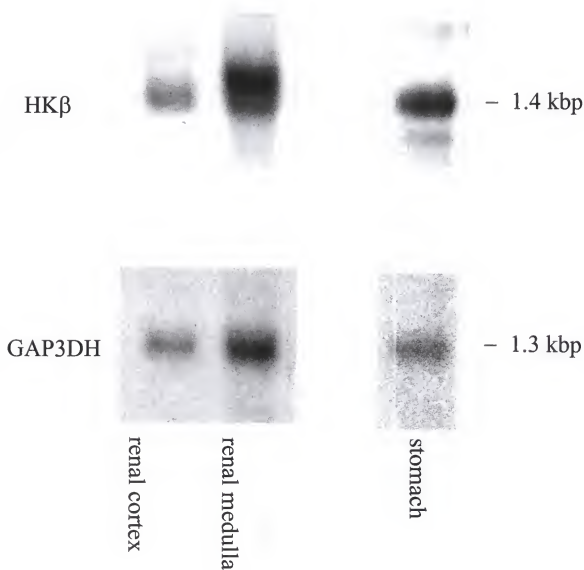


Figure 3-1. Northern analysis showing presence of HK β mRNA in renal cortex, renal medulla, and stomach. The existence of two mRNA species was clearly visible in renal medulla, whereas a single mRNA species was detected in stomach and renal cortex. GAP3DH was used to show the condition of the RNA samples.

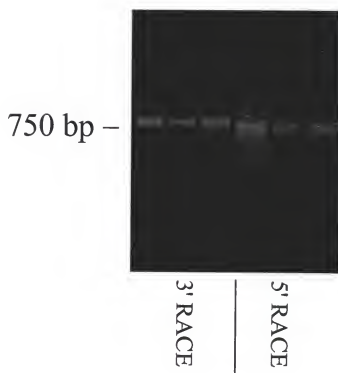


Figure 3-2. 3' and 5' RACE reactions to amplify HK β cDNAs using rabbit renal medulla RNA as template.

the published HK β cDNA sequence. However, all ended approximately 20 bp from the 5' end of the published sequence making it impossible to confirm the extreme 5' end of the sequence.

One of the sequences had a very different 5' end than the others. In that sequence (Figure 3-3A), the first 11 bp of the published HK β sequence was not found, and in its place there was a different 118 bp region (Figure 3-3B). The sequence was confirmed by 5' RACE using a primer specific to the variant extension (CCCTGCACCCCGACTGAGG, nucleotides 104-121, BC388).

To examine the possibility that this renal medulla-specific transcript might be regulated by K⁺ status in rabbits, northern analysis was carried out using total RNA from animals fed a low K⁺ diet. Renal medulla total RNAs from four rabbits fed a Harlan (Indianapolis, IN) control diet and four rabbits fed a Harlan K⁺-restricted diet for two weeks were probed to visualize HK β subunits using a probe made from 570 bp of coding region (Table 2-1). Neither of the two HK β transcripts of renal medulla had systematic variation in HK β mRNA level due to K⁺ restriction in these animals. Northern analysis of renal cortex and stomach RNA from these same rabbits showed uniform level of HK β mRNA between rabbits. No regulation of HK β was observed, although in renal medulla there was a great deal of individual variation between rabbits.

Discussion

A rabbit renal medulla-specific HK β variant mRNA was cloned by 5' RACE and sequenced. It was not known why only one out of the ten sequences proved to be a

A)

```

1  GGAGCTGATGGCTGCTGCTGATAGCACCGCCTCGAGCCAGCCCTGCAGGCGTCGCCGCGATGCCCTTTGACCGTGGCCGG  80
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
81  GGGAGGCTATAAGACCCAGGGGGCCTCCGGCCTCAGTCGGGGTGCAGGGTGGGGGAGCGGCGGCTTCCACAGCAGACACC  160
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

161  ATGGCCGCCTTGCAGGAGAAGAAGTCGTGCAGCCAGCGCATGGAGGAGTTCCGCCACTACTGCTGGAACCCGGACACGGG  240
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
1  M  A  A  L  Q  E  K  K  S  C  S  Q  R  M  E  E  F  R  H  Y  C  W  N  P  D  T  G  27

241  ...

28  ...   Remainder of sequence omitted for clarity

```

B)

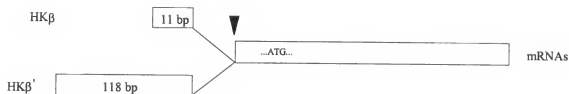


Figure 3-3. HKβ and β' subunits. A) The 5' end of the HKβ' cDNA and its deduced amino acid sequence. Numbering begins at the start of the HKβ' transcript. HKβ' nucleotide 119 corresponds to nucleotide 12 of published gastric HKβ sequence (Reuben, *et al.*, 1990), and the point of divergence is marked by ▼. B) Comparison of HKβ and HKβ' mRNAs. There is no difference in the deduced amino acid sequence.

variant, since both transcripts are observed by northern analysis at comparable levels. Perhaps the extra length or RNA secondary structure reduced amplification efficiency. Because the variant 5' end differed in sequence from the 5' end reported for rabbit (Reuben *et al.*, 1990), the variant is presumed to arise from alternative splicing. This was not pursued because our focus was on the H,K-ATPase α isoforms. HK β mRNA and HK β ' mRNA produced identical HK β subunit proteins. The splice sites of the related rat and mouse HK β genes (Newman and Shull, 1991; Canfield and Levenson, 1991) are not conserved, and working out this issue might have been a considerable target for our primary emphasis.

The deduced amino acid sequence is unchanged by the variant 5' end. Differential regulation by alternate promoters may be the functional reason for the two transcripts. K⁺ restriction was studied as a possible stimulus for regulating the promoters, but K⁺ status alone did not correlate with differential regulation of the two transcripts. Other stimuli, such as aldosterone levels, may regulate these transcripts in renal medulla. It is interesting to note that although HK β subunit is expressed at extremely high levels in gastric tissues, this variant transcript was not observed in stomach. The tissue-specificity of the variant is striking. Study of its promoter region may be fruitful in terms of finding the cis acting factors that turn the gene on specifically in renal medulla.

H,K-ATPASE α SUBUNITS IN THE RABBIT RENAL CORTICAL COLLECTING DUCT

Introduction

H,K-ATPase has been shown to play a role in acid/base and K^+ transport in kidney collecting duct (for review, see Wingo and Cain, 1993; Wingo and Smolka, 1995). However, many questions remained to be addressed, such as finding which H,K-ATPase isoforms are present in kidney, and determining their distribution along the nephron and collecting duct. This included defining the cell type specificity of each isoform. For many years, the rabbit has been the archetypal experimental animal for use in experiments involving microperfusion of renal tubules. To take advantage of the extensive knowledge of rabbit renal physiology in exploring H,K-ATPase function at the molecular level, primary structure information was needed for H,K-ATPase subunits in that species. Because of the discovery of H,K-ATPase catalytic subunit isoforms in addition to the gastric isoform in several species, the relationship between these isoforms needed to be examined. Perhaps most importantly, the role that the multiplicity of H,K-ATPase subunit isoforms plays in kidney function should be examined.

At the time the experiments described here were undertaken, little was known of the identity of H,K-ATPase subunits in the kidney. mRNAs encoding catalytic subunit isoforms cloned from human axillary skin (Modyanov *et al.*, 1991) and rat distal colon (Crowson and Shull, 1992) were the first to be detected in kidney. In collaborations

including the Cain and Wingo laboratories, HK β (Callaghan *et al.*, 1995, Campbell-Thompson *et al.*, 1995) was observed in rabbit kidney. The key experiment identifying HK β in the kidney dated from 1992, prior to the arrival of this author, so that information was available in designing this dissertation project. As this work progressed, it was found that the HK α_1 subunit isoforms are present in renal tissues (Ahn and Kone, 1995). In addition, an alternatively spliced isoform of rat HK α_2 was recently reported in kidney (Kone and Higham, 1998). The rat HK α_2 alternatively spliced variant was designated HK α_{2b} , with the original renamed HK α_{2a} . The previous experiments were not designed to discriminate between HK α_{2a} and HK α_{2b} .

In these studies a full-length cDNA sequence was determined for the HK α_2 subunit in rabbit. An alternatively spliced variant of this HK α_2 subunit isoform was found in rabbit. The pattern of splicing was identical to that found in rat (Kone and Higham, 1998). However, the translation start site was not conserved. For this reason, the alternatively spliced variant in rabbit was designated HK α_{2c} . Anti-peptide polyclonal antibodies were raised and used to show expression of both HK α_{2a} and HK α_{2c} proteins in rabbit renal cortex. These results allude to a potentially complex pattern of regulation of H,K-ATPase activity in renal cortex.

Multiple H,K-ATPase α Subunits in the Kidney

We set out to find the HK α subunits that mediate the H,K-ATPase activities that had been observed in kidney. Because HK α_{2a} mRNA had been observed in kidney, it was necessary to find the rabbit sequence of HK α_{2a} in rabbit for use in designing tools for

studying H,K-ATPase function in the kidney. Rabbit HK α_1 sequence was known (Bamberg *et al.*, 1992). In designing an experimental approach to this goal, we wanted to consider 1) the possibility that HK α_1 was expressed in kidney (unknown at the time) in addition to HK α_2 , 2) the possibility that the HK α_2 mRNA might be different in kidney than in the other tissues for which full length sequence was known, and 3) that novel HK α isoforms might be present in renal tissues. A good technique to address all these possibilities was to do RT-PCR using degenerate primers designed to anneal to regions of sequence that were highly conserved among P-type ATPases. This approach was expected to amplify any P-type H,K-ATPase that might be present in rabbit kidney.

The aspartyl residue that is phosphorylated as an intermediate in the catalytic cycle of H,K-ATPase lies within a motif that is highly conserved in the entire P-type ATPase family. Another well-conserved region among P-type ATPases surrounds a lysine residue that can be modified by FITC. FITC competes with ATP for binding (Jackson *et al.*, 1983; Farley and Faller, 1985), so this region is thought to make up part of the ATP binding site. Advantage was taken of these two regions to design degenerate primers to amplify any P-type ATPase using renal cortex RNA as template. Fifteen P-type ATPase sequences were aligned using the computer program PILEUP (Genetics Computer Group, 1997). These sequences were selected to give a wide range of mammalian Na,K-ATPases and H,K-ATPases. Chicken Na,K-ATPases were included because all three α subunit isoforms were known for that species so they comprised a good example of P-type ATPases from an organism less related to mammals. Toad sequences were included because toad

H,K-ATPase was one of only three non-gastric H,K-ATPases known at the time.

Degenerate primers were then designed to amplify any of these ATPases (Figure 4-1).

The products of the first two RT-PCR reactions using these primers, when cloned and sequenced, had high homology to the NaK α_1 subunit in other species. The third reaction product (Figure 4-2) was more related to the non-gastric H,K-ATPase α subunits than to the HK α_1 subunit or to any of the Na, K-ATPase α subunits. It was a 419 bp fragment that corresponded to nucleotides 1182-1600 of the full-length rabbit HK α_{2a} cDNA sequence. A BLAST search listed three non-gastric H,K-ATPases as the most highly aligned sequences to the 419 bp sequence, with other H,K-ATPases and Na,K-ATPases being less well aligned (Figure 4-3). The sequence of the fragment shared 89% nucleic acid identity with the rat distal colonic H,K-ATPase α subunit, 88% with a guinea pig distal colonic H,K-ATPase α subunit, and 86% with the H,K-ATPase α subunit cloned from human axillary skin. Based on these similarities, this fragment was tentatively identified as belonging to an H,K-ATPase.

Using degenerate primers designed to anneal to conserved sequences we identified farther 5' and 3' in the sequence alignment paired with gene-specific primers designed based on the new sequence (Table 2-1), fragments containing more of the transcript were cloned (Figure 4-4). Outside the coding sequence, homology between cDNA sequences declines. With much of the coding region cloned and sequenced, 5' and 3' RACE were used to clone the full extent of the cDNA including the two ends. The 3' end was easily obtained; a single 3' RACE product was observed the first time the procedure was performed. The 5' end proved far more interesting, but more challenging to find. Initial

A)

```

Hsu02076      ...CCATCATCTGCTCGGACAAGACTGGGACAC...
Ratatpasez    ...CCATCATCTGCTCAGACAAGACGGGGACCC...
Bmhkatpas     ...CCATTATCTGCTCCGACAAAACAGGAACCC...
Doghkatp      ...CAGTGATCTGCTCAGACAAGACAGGGACCT...
Pigatphk       ...CAGTCATCTGCTCTGACAAGACGGGGACCC...
Ocatprna      ...CGGTGATCTGCTCCGACAAGACGGGGACCC...
Ratatpast     ...CAGTCATCTGCTCAGACAAGACAGGAACTC...
Chknakat2     ...CCACCATCTGCTCCGACAAAACCGGGACCC...
Chknakat3     ...CCACCATCTGCTCCGATAAGACCGGGACCC...
Ratatpa3      ...CCACCATCTGCTCCGACAAGACCGGCACCC...
Ratatpa2      ...CCACCATCTGCTCCGACAAGACAGGCACCC...
Ratatpa1      ...CCACCATCTGCTCCGACAAGACTGGAACCT...
Chkatpas      ...CCACCATCTGTTCTGACAAAACAGGCACCC...
Bmnkaa1       ...CCACCATCTGCTCTGACAAGACCGGAACCC...
Tcatpmr       ...CAACCATTTGCTCAGACAAAACCTGGAACCT...

```

5' Primer

```

          A      A
ATCTGCTCCGACAAAACCGG
          G      G
          T      T

```

B)

```

Hsu02076      ...TCATGGTGATGAAGGGGGCCCCCTGAGCGCA...
Ratatpasez    ...TCGTGGTGATGAAAGGAGCCCCCTGAGAGGA...
Bmhkatpas     ...TGCTCGTCATGAAAGGTGCCCCAGAGAGA...
Doghkatp      ...TGCTGGTGATGAAGGGCGCCCCGAGCGCG...
Pigatphk       ...TGCTTGTGATGAAGGGCGCCCCGAGCGCG...
Ocatprna      ...TGCTGGTGATGAAGGGCGCCCCGAGCGCG...
Ratatpast     ...TGCTGGTGATGAAGGGCGCCCCAGAGCGCG...
Chknakat2     ...TCCTGGTGATGAAAGGGGGCCCCGAGCGCA...
Chknakat3     ...TGCTGGTGATGAAAGGGCGCCCCGAGCGCA...
Ratatpa3      ...TGTTAGTGATGAAGGGCGCCCCGAACGCA...
Ratatpa2      ...TGCTGGTGATGAAAGGTGCCCCGAGCGCA...
Ratatpa1      ...TGCTAGTGATGAAGGGCGCCCCAGAAAGGA...
Chkatpas      ...TGCTGGTGATGAAGGGGAGCTCCAGAGAGGA...
Bmnkaa1       ...TGCTGGTCATGAAGGGCGCCCCGAGAGGA...
Tcatpmr       ...TGTTGGTGATGAAGGGAGCACCAGAACGGA...

```

3' Primer

```

          A      A
GTGATGAAAGGCGCCCCGA
          G      G
          T      T

```

Figure 4-1. Design of degenerate primers for RT-PCR of novel P-type ATPases. A) Upstream primer was designed to anneal to well-conserved sequence at the enzyme active site phosphorylated aspartyl residue. GenBank loci of 15 aligned sequences are shown at left. Oligonucleotide is shown beneath aligned sequences. B) Downstream primer was designed to anneal to well-conserved sequence at the putative ATP binding site. Oligonucleotide is reverse complement of consensus sequence shown.

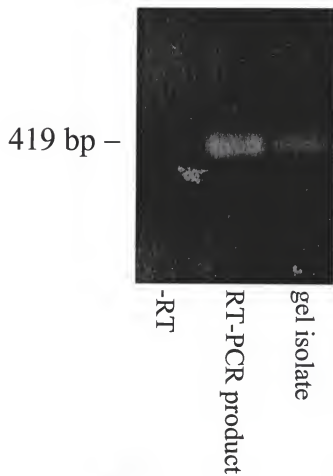


Figure 4-2. RT-PCR product amplified from rabbit renal cortex RNA using degenerate primers. Gel isolated product was cloned and sequenced.

Sequences producing High-scoring Segment Pairs:			Smallest Sum	
			Score	P(N)
gb U02076	HSU02076	Human ATP-driven ion pump (ATP1A1L...	1663	4.1e-128
gb M90398	RATATPASEZ	Rat H ₂ K ₂ -ATPase mRNA, complete cds.	1582	2.2e-121
gb U94912	RNU94912	Rattus norvegicus H-K-ATPase alpha...	1582	2.2e-121
dbj D21854	GPIHKAAS	Guinea pig mRNA for distal colon H...	1582	2.2e-121
gb U94913	RNU94913	Rattus norvegicus H-K-ATPase alpha...	1582	2.2e-121
emb Z25809	BMHKATPAS	B.marinus mRNA for H,K-ATPase	1069	1.1e-78
gb M59960	CHKNAKAT3	Chicken Na,K-ATPase alpha-3-subuni...	907	3.1e-65
emb X05883	RNATPAHO	Rat mRNA homologous to alpha subun...	898	1.8e-64
gb M14513	RATATPA3	Rat Na ⁺ , K ⁺ -ATPase alpha(III) isof...	898	1.8e-64
gb M28648	RATNALPH2	Rattus norvegicus Na,K-ATPase alph...	889	9.9e-64
emb Z11798	BMNKAAL	B.marinus mRNA for Na, K-ATPase al...	889	9.9e-64
gb U10108	XLU10108	Xenopus laevis Na ⁺ -K ⁺ -ATPase alpha...	880	5.5e-63
gb U49238	XLU49238	Xenopus laevis adenosine triphosph...	871	3.1e-62
gb J02649	RATATPAST	Rat stomach (H ₂ K ₂)-ATPase mRNA, c...	853	9.7e-61
gb U17249	XLU17249	Xenopus laevis gastric H(+)-K(+)-A...	844	5.5e-60
emb X64694	OCATPRNA	O.cuniculus mRNA for ATPase (alpha...	835	3.1e-59
gb J03230	CHKATPAS	Chicken (Na ⁺ + K ⁺)-ATPase mRNA, co...	826	1.7e-58
gb S66043	S66043	sodium pump alpha subunit (Ctenoce...	826	1.7e-58
gb U17282	MMU17282	Mus musculus gastric H(+)-K(+)-ATP...	817	9.6e-58
gb L42565	HUMATP1G04	Homo sapiens (clone lSW34) non-gas...	458	2.4e-56
gb L11568	DOGHKATP	Dog H ₂ K ₂ -ATPase mRNA, complete cds.	790	1.7e-55
emb X02813	ORATPMR	Sheep mRNA for (Na ⁺ and K ⁺) ATPase...	772	5.3e-54
gb M22724	PIGATPHK	Pig (H ⁺ + K ⁺)-ATPase mRNA, complet...	772	5.3e-54
gb M28647	RATNALPH1	Rattus norvegicus Na,K-ATPase alph...	763	3.0e-53
gb S74801	S74801	H(+)-K(+)-ATPase alpha-subunit (ra...	760	5.3e-53
gb M14511	RATATPA1	Rat Na ⁺ ,K ⁺ -ATPase alpha isoform ca...	736	5.3e-51
gb M74494	RATNAKATP	Rat sodium/potassium ATPase alpha...	736	5.3e-51
emb X76108	AASPA	A.anguilla mRNA for sodium/potassi...	727	2.9e-50
emb X05882	RNATPAR	Rat mRNA for alpha subunit kidney...	727	2.9e-50
emb X02810	TCATPMR	Torpedo californica mRNA for (Na ⁺ ...	667	3.9e-49
gb M59959	CHKNAKAT2	Chicken Na,K-ATPase alpha-2-subuni...	710	7.6e-49
gb U16798	HSU16798	Human Na,K-ATPase alpha-1 subunit ...	709	9.3e-49
emb X04297	HSATPAR	Human mRNA for Na,K-ATPase alpha-s...	709	9.3e-49
gb J03007	HUMATPAS	Human Na ⁺ ,K ⁺ ATPase alpha-subunit ...	709	9.3e-49
gb L42173	DOGNKAA	Canis familiaris Na, K-ATPase alph...	700	5.2e-48
gb M38445	PIGATPBSEN	Pig Na ⁺ , K ⁺ -ATPase alpha subunit m...	691	2.9e-47
emb X03938	SSATPAR	Pig mRNA for (Na ⁺ , K ⁺)-ATPase alph...	682	1.6e-46
gb M14512	RATATPA2	Rat Na ⁺ ,K ⁺ -ATPase alpha(+) isoform...	674	7.6e-46
emb X58629	CCNAKATP	C.commersoni mRNA for Na(+)/K(+) A...	665	9.0e-46
emb X56650	AFNAKATP	A.franciscana mRNA for Na/K ATPase...	646	1.6e-43
gb M75140	HYDATPASE	H.vulgaris Na,K-ATPase alpha subun...	606	3.4e-40
gb L42566	HUMATP1G05	Homo sapiens (clone lSW11-1) non-g...	551	1.3e-35
gb S76581	S76581	Na,K-ATPase alpha-1 subunit [dogs,...	514	1.5e-32
gb J05451	HUMATPGG	Human gastric (H ⁺ + K ⁺)-ATPase gen...	318	4.8e-24
gb M63962	HUMHKATPC	Human gastric H,K-ATPase catalytic...	327	5.5e-24

Figure 4-3. BLAST search using sequence of 419 bp fragment of HK α_2 .

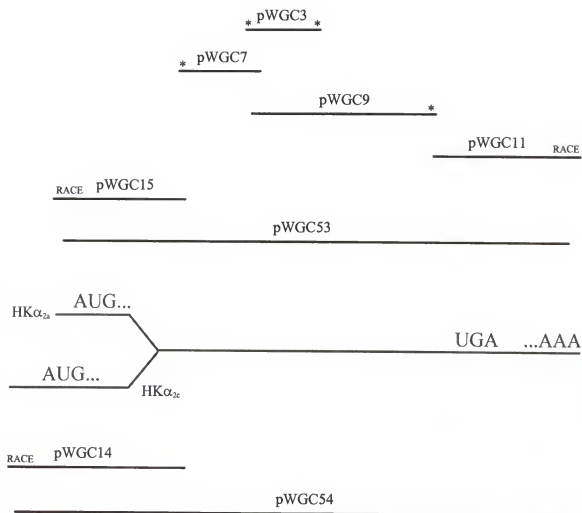


Figure 4-4. Cloning of HK α_{2a} and HK α_{2c} cDNAs. Individually cloned cDNAs are indicated by bars and the plasmid numbers are indicated. AUG and UGA indicate start and stop codons of the mRNA, respectively. Symbols: *, degenerated primers; RACE, RACE primer. All other primers were gene-specific.

attempts employed the classical technique of Frohman *et al.* (1988), which is analogous to the 3'RACE process. In several attempts, this technique produced only short cDNA sequences which provided little new sequence information. Taking a new approach, the Marathon cDNA Amplification Kit by Clontech (Palo Alto, CA) was tried. This kit uses a ligation technology to add a known upstream sequence for PCR amplification, rather than the less efficient terminal transferase technology employed by Frohman *et al.* (1988). Also, the reverse transcription step was changed to a higher temperature to lessen the possibility of secondary structure blocking full extension by the RNA polymerase. With the new protocol, after some optimization, two different 5' ends were obtained when 5' RACE products were sequenced.

Two rabbit renal HK α_2 cDNA sequences were found in this manner, having 4035 bases in common at the 3' end but different 5' ends. The GenBank records of these two cDNAs are shown in Figure 4-5. A segment of the shared 3' portion of the sequences was identical to the 1456 bp sequence obtained by Fejes-Tóth (1995) except for two single base mismatches. One mismatch was a transition of C→T at nucleotide 2927, which does not change the primary protein sequence and the other a transversion of G→T at nucleotide 3259, in the 3' untranslated region. The full-length rabbit HK α_{2a} nucleotide sequence was 86% identical to human HK α_2 and 83% to rat, whereas identity of HK α_{2a} to rabbit HK α_1 was only 67%. The deduced amino acid sequence shared 87% identity with human HK α_2 , 87% identity with rat HK α_2 , but merely 64% with rabbit HK α_1 . Northern analysis using an HK α_{2a} -specific probe (Table 2-1) is shown in Figure 4-6.

A)

LOCUS AF023128 4079 bp mRNA MAM 13-OCT-1997
 DEFINITION *Oryctolagus cuniculus* H⁺,K⁺-ATPase alpha 2a subunit mRNA, complete cds.
 ACCESSION AF023128
 NID g2511766
 KEYWORDS .
 SOURCE *Oryctolagus cuniculus*.
 ORGANISM *Oryctolagus cuniculus*
 Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia;
 Eutheria; Lagomorpha; Leporidae; *Oryctolagus*.
 REFERENCE 1 (bases 1 to 4079)
 AUTHORS Campbell,W.G., Weiner,I.D., Wingo,C.S. and Cain,B.D.
 TITLE H,K-ATPase in the RCCT-28A rabbit cortical collecting duct cell line
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 4079)
 AUTHORS Campbell,W.G., Wingo,C.S. and Cain,B.D.
 TITLE Direct Submission
 JOURNAL Submitted (08-SEP-1997) Biochemistry, University of Florida, JHMH 100245, Gainesville, FL 32610, USA
 FEATURES Location/Qualifiers
 source 1..4079
 /organism="Oryctolagus cuniculus"
 /strain="New Zealand White"
 /db_xref="taxon:9986"
 CDS 39..3140
 /note="P-type ATPase"
 /codon_start=1
 /product="H⁺,K⁺-ATPase alpha 2a subunit"
 /db_xref="PID:g2511767"
 /translation="MRQRKLEIYSVELHAATDIKKKEGRDGKKDNDLELKRNVQKEEL
 KKELDLDDHKLNSKELETKYGTDIIRGLSSTRAAELLAQNGPNALTPPKQTPEIKFL
 KQMVGGFISILLWVGAVLCWIAFGIQYVSNPSSASLDRVYLGTVLAVVVILTGIFAYYQE
 AKSTNIMASFCKMIPQQAUVIRDSEKKVIPAEQLVVGDIVIIEKGGDQIPADIRLLSAQ
 GCKVDNSSLTGESEEPQSRSSGFTHENPLETKNITFYSTTCLEGTATGMVINTGDRITII
 GRIASLASGVGNEKTPIAIEIEHFVHIVAGVAVSVGILFFIIAVCMKYHVLDAIIFLI
 AIIIVANVEGLLATVTVALSLTAKRVAKKNCLVKNLEAVETLGSTSIICSDKTKTLTQ
 NRMVTVAHLWFDNQIFVADTSEDNLNQGFDQSSGTWTSLSKIIALCNRAEFKPGESV
 IMKRVVVGDASETALLKFSEVILGDVMEIRKRNHKVVEIPFNSTNKFQLSIHQTEDPN
 DKRFLVMKGAPERILEKCESTIMINGKEQPLDKSMAQAFHTAYMELGGLGERVLGFCF
 FYLPADFEPPETYSFDSSESMNFPSTNLNCFVGLLSMIDPPRSTVPDAVTKCRSAGIKVIM
 VTGDHPITAKAIAKSVGIIISANSETVEDIAKRCNIAVEQVKNRDKAAAVVTGMELKDM
 SPEQLDELLANYPEIVFARTSPQQKLIIEVEGQQRQDAVVAVTGDGVNDSPALKKADIG
 VAMGITGSDAAKNAADMILLDDNFSSIVTGVEEGRILFDNLKKTIAVTLTKNIAELCP
 FLIYIILGLPLPIGTITLLFDLGTDIIPSIALAYEKAESDIMNRKPRHKKKDRLVNQ

Figure 4-5. GenBank accession records for rabbit HK α_2 sequences. A) Genbank record for rabbit HK α_{2a} . B) Genbank record for rabbit HK α_{2c} .

QLAVYSYLHIGLMQALGAFVYFTVYAQQGFRPTSLFHLRIAWDSHLDNDLEDNYGQE
 WTSYQRQYLEWTGYTAFFVGMVQQIADLIIRKTRKNSIFKQGLFRNKVIWVGIASQI
 IVALLSYGLSGSITALNFTMLKAQYWFVAVPHAILIWVYDEMRLKFIIRLYPGSSWWDKN
 MYV"

BASE COUNT 1037 a 1073 c 1024 g 945 t

ORIGIN

```

1 gccccctgcc gcgcgaccgc cgcgccctcc agcgcgacat gcgcgcagaga aagctggaaa
61 tttactccgt ggagctccat gcagctacag atatcaagaa gaaggagggg cgagatggca
121 agaaagacaa tgacttggaa ctcaaaagga atcagcagaa agaggagctt aagaaaagaac
181 ttgatctgga tgaccacaaa ctacgaata aggagctgga aacgaatatat ggcacagaca
241 tcattcgggg tctctccagc accagagctg ctgagctcct ggcacagaaac ggaccacaacg
301 ccttcacccc tcccaaacag accccagaga tcatacaagt cctcaagcag atggtgggcg
361 gcttttccat ccttctgtgg gtaggagctg tcctgtgttg gatcgattt gggattcagt
421 atgtcagcaa tccatctgcc tccctggaca gagtgtacct gggcaactgta cttgcgctgg
481 ttgtcatttt aacaggaatc ttgtcctatt accaagaggg aaaaagcacc aacatcatgg
541 ccagcttctg caagtgatc cccagcaag ctgttgtcat ccgtgactcg gagaaaaagg
601 ttatccctgc agagcagctg gtggtggggg acatcgtgga gattaaagga ggtgaccaga
661 ttctctgcga catcagctg ctgcttgccc aggggtgtaa ggtggataac tcattcttta
721 ctggagagtc tgagcccgag tcccgctcaa gtgggttcc ccaagaaaaa cccctggaaa
781 caaagaacat cactttctac tccacgacct gcctggaagg cactggttca ggcattggtc
841 tcaacacggg tgacgggacc atcattggcc gcattgcctc ctgtgcttca ggcgtcgga
901 atgagaagac gccattgcc attgagatcg aacatttgtt gcacatttgt gcaggagtgg
961 ccgtctccgt cgccatcctg ttcttctaca tcgcagtgtg catctgtctc cactgctctg
1021 acgccaatcat ctctctcatt gccatcattg tggccaaact gcctgaaggc ctctggcca
1081 ctgtcactgt ggcctgtctg ctacacagcca aacgggtggc caagaagaac tgctctggtg
1141 agaacttggg ggcagtgga accctcggtc ccaactccat catctgtctc caagaactg
1201 ggaactcgac gcgaacagg atgacccgtg cccatctgtg gtttgacaaat cagatcttcg
1261 tggccgacac gagtgaagac aatttaaacc aaggtcttga ccaagctctt ggaacctgga
1321 cctccttgtc caagataata gcattgtgta accgagctga gttcaagcca ggagaggaga
1381 gtgtccccat catgaagaga gtctgtgttg gagatgcttc agaaactgct ctctgaaat
1441 tctcagaagt cattttgggt gacgtgatgg aaattagaaa aagaaccacc aaagtagtgc
1501 aaatcccttt taactcaacc aacaaatttc agctctccat acaccagacg gaagatccca
1561 atgacaagcg ctctctgctg gtgatgaagg gggcccccga gcggatccca gagaagtgc
1621 gaccatcat gatcaacggc aaggagcagc cactggacaa gagcatggcc caggcccttc
1681 acacggccta catggaactg ggcggcctgg gcgagcgctg ctctgggttc tgccatttct
1741 acctgccagc agatgagttt ccagagacct actcatttga ctacagaatcc atgaacttcc
1801 ccacctccaa ctctatgttt gtggggctct tatcaatgat tgcctctcct cgactcactg
1861 tccagatgac agtcaccaaa tgccggagtg caggaaatcaa gttatcagtg gttacagtg
1921 atcatcccat cacagccaaa gccattgcca agagtgtagg gatcatttca gccaacagt
1981 agacagtgga agacatttga aaacgctgca acatcgccgt ggagcaggtt aacaaacggg
2041 atgccaaagg cgccgtgggt accggcatgg agctgaagga gactgaagga caacagctgg
2101 atgagctctt agccaactac ccggaatcgc tgtttgcacg gacgtccccc cagcaaaagc
2161 tgatcatcgt ggagggtctg cagaggcagg acgcagttgt ggcctgtgac ggggacggag
2221 tgaatgactc ccccgtctta aagaaggcgc acattggctg tgccatgggg ataacgggtt
2281 ctgacgcggc caagaacgca gccgacatga tcctgtctga tgacaacttc tcctctatcg
2341 tcacaggggt ggagggaagg cgcttgatat ttgacaacct aaagaagacc atcgcttaca
2401 cctgaccaaa gaacattggc gagctctgcc ccttttgtat ttacatcatt ctcgggctgc
2461 cctgccccat tggcaccatc accctcctgt tcactgactt gggcacagac ataatccct
2521 ccattgctt ggcgctatgg aaagcagaaa gtgacattat gaacaggaag cctcggcaca
2581 agaaaaagga cagactgggt aaccagcagc ttgctgtata ctactcctg cacatttgcc
2641 tcatgcaagc cctgggagct ttctgtgtgt acttcaactg gtacgcacag cagggtcttc
2701 ggccgacctc actgtttcac ctgcggatag cgtgggacag cgaccactg aacgacttgg

```

Figure 4-5--continued

```

2761 aagacaacta tggacaggaa tggacgagtt atcagaggca atacctggaa tggacaggct
2821 acacggcttt ctttgttggc atcatggtcc agcaaatagc agatctgac atcaggaaga
2881 cccgcaagaa ctccatcttc aagcaggggc tcttcagaaa taaagtatc tgggtgggga
2941 tcgectcca gatcatcgtc gccctgctcc tctcttacgg gctcggcagt atcacagccc
3001 taaatttcac catgctcaag gctcagtact ggtttgtggc cgtacccac gccatcctga
3061 tctgggtata cgatgaaatg cggaaactct tcatcaggct ctaccccga agctggtggg
3121 ataagaacat gtattactga gaccaggtct gtctctgagt ctcccagcg cacctgcctg
3181 gtggctctcg gcaagacctc tgtgtagtgt ggatgttggc aagctccact cgggaggaga
3241 ctctcatcta gaacacagtg gtgaagcttc ttactgatct gttgtacttc aaagctgaga
3301 ttcagctggt tgtatatgat ttcatctct atctccatct ccttacctta aaagatgtgg
3361 atgtcaaggt catggtgtag ggaaggatgt gtttatctgt atatgaagct cactgatgtc
3421 acacagactt gtgtaaccca ggtggctgct ggagtctgcc ataagttgag ctagaattgc
3481 tcagatctcc ttccacacc ttgcaaaggc ccggtgagct ccataggatt tctgtgaatc
3541 cccctgaaac ataacttttg gggtttgctt tgctcagctg aggggtgtgag ttggaagtgt
3601 ggcagcagga gcacctcaga acagcaaaga cagccccctg tttgactccc agacactttg
3661 ttgctgtgat gggttcctgg ccatgcggcc ccagtccgcc ttctcacage actccaccac
3721 ctgttctctgc aaagctgacc tccaagtcca ttccacaaa ctttaactca acattcgtgg
3781 acccaaaggg gctgtcactg actgggactc ggctctccg gaaagccact gtggtttaga
3841 tagcactatt tatttcttgt agataggctg ccaagcactc tccagcagcc attttatgtc
3901 aatcacattt ttgtaactta gatataattg tgtgggacac gaaacacata catccatgtt
3961 gacaggtttt tttttttaa taaaagatgt ttttaagtaa aatgttttat gaaacaaaat
4021 ctaattgtga tgttttactt aattcaagtt ttccagag caggcacgga aaataccaa

```

//

B)

LOCUS AF023129 4422 bp mRNA MAM
 16-APR-1998
 DEFINITION *Oryctolagus cuniculus* H+,K+-ATPase alpha 2c subunit mRNA, complete cds.
 ACCESSION AF023129
 NID g2511768
 KEYWORDS .
 SOURCE *Oryctolagus cuniculus*.
 ORGANISM *Oryctolagus cuniculus*
 Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia;
 Eutheria;
 Lagomorpha; Leporidae; *Oryctolagus*.
 REFERENCE 1 (bases 1 to 4422)
 AUTHORS Campbell,W.G., Weiner,I.D., Wingo,C.S. and Cain,B.D.
 TITLE H,K-ATPase in the RCCT-28A rabbit cortical collecting duct cell line
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 4422)
 AUTHORS Campbell,W.G., Wingo,C.S. and Cain,B.D.
 TITLE Direct Submission
 JOURNAL Submitted (08-SEP-1997) Biochemistry, University of Florida, JHMH
 100245, Gainesville, FL 32610, USA
 FEATURES Location/Qualifiers
 source 1..4422
 /organism="Oryctolagus cuniculus"
 /strain="New Zealand White"
 /db_xref="taxon:9986"
 CDS 199..3483
 /note="P-type ATPase"
 /codon_start=1
 /product="H+,K+-ATPase alpha 2c subunit"
 /db_xref="PID:g2511769"
 /translation="MAGGAHRADRTGEERKEGGRRWRAPHSPSPPGPRGCPVLKAA
 AQS LCRKPTWGRYCTLLLFQRKLEIYSVELHAATDIKKKEGRDGKKDNDLELKRNQK
 EELKKELDLDDHKLSNKELETKYGTDIRGLSSTRAAELLAQNGPNALTTPKQTPETI
 KFLKQMVGGFSILLWVGAVLCWIAFGIQYVSNPSASLDRVYLGTVLAVVVILTGFAY
 YQEA KSTNIMASFCKMIPQQA VIRDSEKKVIPAEQLVVG DIVEIKGGDQIPADIRLL
 SAQ GCKVDNSSLTGESE PQSRSSGFTHENPLETKNITFYSTTCLEGTATGMVINTGDR
 TIIGRIASLASGVGNEKTPIAIEIEHFVHIVAGVAVSVGILFFIIAVCMKYHVLDAII
 FLIAIIVANVPEGLLATVTVALSLTAKRVAKKNCLVKNLEAVETLGSTSIICSDKTGT
 LTQNRMTVAHLWFDNQIFVADTSEDNLNQGFDQSSGTWTSLSKIIALCNRAEFKPGEE
 SVPIMKR VVVGDASETALLKFSEVILGDVMEIRKRNHKVVEIPFNSTNKFQLSIHQTE
 DPNDKRFLVLNKGAPERILEKCS T IMINGKEQPLDKSMAQAFTAYMELGGLGERVLG
 FCHFYLPADEFPETYSFDSSESMNFPTSNLCFVGLLSMIDPPRSTVPDAVTKCRSAGIK
 VIMVTGDHPITAKAIAKSVGIIISANSETVEDIAKRCNIAVEQVNRKDAKAAVVTGMEL
 KDMSPEQLDELLANYPEIVFARTSPQOKLIIIEGCQRQDAVVAVTGDGVNDSPALKKA
 DIGVAMGITGSDAAKNAADMILLDDNFSSIVTGVEEGRILFDNLNKKTIAYTLTKNIAE

Figure 4-5--continued

LCPLFIYIILGLPLPIGTITLLFIDLGTDIIPSIALAYEKAESDIMNRKPRHKKKDRIL
VNQQLAVYSYLHIGLMQALGAFVYFTVYAQQGFRPTSLFHLRIAWDSHLDNLEDNY
GQEWTSYQRQYLEWTGYTAFFVGMVQQIADLIIRKTRKNSIFKQGLFRNKVIVWGIA
SQIIVALLSYYGLGSITALNFTMLKAQYWFVAVPHAILIWWYDEMRLKFLIRLYPGSWW
DKNMYV"

BASE COUNT 1103 a 1174 c 1136 g 1009 t

ORIGIN

1	ctccgccctc	gcacctgcgg	gctcggattc	ggagaaaaagt	gctagactgg	agctacacgt
61	atcgctagcg	gtctggaaaa	tgccccaggc	tcgggtctga	ggggcccaag	tctatgcacc
121	gctgggtgtga	ccccgcaggg	caaccccgcg	gttaacttct	ctcctgccca	ccccatagagg
181	tgtcttctcg	ggaagacgat	ggcaggcggt	gcccaccgag	ccgaccgtgc	aacaggggaa
241	gagaggaagg	agggaggtgg	gaggtggcgc	gctccccaca	gcccttcccc	tcttggcccc
301	cgaggggtgtc	cggtcccact	caaggcagct	gcgcagagcc	tgtgcagaaa	accaccatgg
361	ggccggtatt	gactcttctg	tctctttcag	agaaagctgg	aaatttactc	cgtggagctc
421	catgcagcta	cagatatcaa	gaagaaggag	gggcgagatg	gcaagaaaga	caatgacttg
481	gaactcaaaa	ggaatcagca	gaaagaggag	cttaagaaag	aacttgatct	ggatgaccac
541	aaactcagca	ataaggagct	ggaacacgaa	tatggcacag	acatcattcg	gggtctctcc
601	agcaccagag	ctgtctgagct	cctggcacag	aacggaccaca	acgcctctac	ccctcccaaa
661	gacaccccg	agatcatcaa	gttcctcaag	cagatggttg	gcggcttttc	catccttctg
721	tgggtaggag	ctgtcctgtg	ttggatcgca	tttgggattc	agtatgtcat	caatccatct
781	gcttcctctg	acagagtgtga	cctgggcact	gtacttgcg	tgttgtgcat	tttaacagga
841	atctttgctc	attaccaaga	ggcaaaaagc	accaacatca	tggccagctt	ctgcaagatg
901	atccccccag	aagctgttgt	catccgtgac	tcggagaaaa	aggttatccc	tgcagagacg
961	ctgggtgggtg	gggacatcgt	ggagattaaa	ggaggtgacc	agattctctg	cgacatcagg
1021	ctgctgtctg	cccagggggtg	taagtggtgat	aactcatctc	ttactggaga	gtctgagccc
1081	cagtcctcgt	caagtggggt	cacccacgaa	aacccctctg	aaacaaagaa	catcactttc
1141	tactcacagc	ctgcctcctga	aggcacggca	actggcatcg	ctatcaaac	gggtcctggg
1201	accatcattg	ggcgcatctgc	ctccttggtc	tcaggcgctg	ggaatgagaa	gacgcccatt
1261	gccatttga	tcgaacattt	tgtgcacatt	gtggcaggag	tggccgtctc	cgctggcatc
1321	ctgttcttca	tcctcgcatg	gtgcatgaag	taccacgtcc	tggacgcat	catcttctcc
1381	attgccatca	ttgtggccaa	cgtgcctgaa	ggcctccttg	ccactgtcac	tgtggccctg
1441	tcgtccacag	ccaaacgggt	ggccaagaag	aactgccttg	tgaagaactt	ggaggcagtg
1501	gagaccctcg	gctccacctc	catcatctgc	cttgacaaga	ctgggaactct	gacgcagaac
1561	aggatgaccg	tggcccatct	gtggtttgac	aatcagatct	tcgtggccga	cacgagttaa
1621	gacaatttaa	accaaggctt	tgaccaaagc	cttggaacct	ggacctctct	gtccaagata
1681	atagcattgt	gtaaccgagc	tgagtccaag	ccaggagagg	agagtgtccc	catcatgaag
1741	agagtcgttg	ttggagatgc	ttcagaaaact	gtcttctaga	gtcttctaga	agtcattttg
1801	ggtgacgtga	tggaaattag	aaaaagaaac	cacaaagtag	tcgaaatccc	ttttaactca
1861	accaacaaat	ttcagctctc	catacaccag	acggaagatc	ccaatgacaa	gcgcttccctg
1921	ctgggtgatga	agggggccccc	cgagcggatc	ctagagaagt	gcgacaccat	catgatcaac
1981	ggcaaggagc	agccacttga	caagagcatg	gcccaggcct	tccacacggc	ctacatggag
2041	ctggggcgccc	tgggcgagcg	cgtgctgggt	ttctgccatt	tctacctgcc	agcagatgag
2101	tttccagaga	cctactcatt	tgactcagaa	tctcagaact	tcccacctcc	caacttatgt
2161	tttgtggggc	tcttatcaat	gattgatcct	cctcgatcca	ctgtcccaga	tgcatgcacc
2221	aaatgccgga	gtgcaggaat	caaggttatc	atgggttacag	gtgatcatcc	catcacagcc
2281	aaagccattg	ccaagagtgt	agggatcatt	tcagccaaca	gtgagacagt	ggaagacatt
2341	gcaaaacgct	gcaacatcgc	cgtggagcag	gttaacaaac	gggatgccaa	ggccgcctgt
2401	gtgaccggca	tggagctgaa	ggacatgagc	ccagaacagc	tggatgagct	cttagccaac
2461	tacccgaaa	tcgtgtttgc	accgacgtcc	ccccagcaaa	agctgatcat	cgtggagggc
2521	tgtcagaggc	aggacgcagt	tgtggccgtg	acgggggacg	gagtgataga	ctcccccgct
2581	ctaaagaagg	ccgacattgg	cgttgccatg	gggataacgg	gttctgacgc	ggccaagaac

Figure 4-5--continued

```

2641 gcagccgaca tgatcctgct ggatgacaac ttctcctcta tcgtcacagg ggtggaggaa
2701 ggcgagcttga tatttgacaa cctaaagaag accatcgctt acaccctgac caagaacatt
2761 gccgagctct gccccctttt gatttacatc attctcgggc tccccctgcc cattggcacc
2821 atcaccctcc tgttcacga ctggggcaca gacataatcc cctccattgc ctggcgctat
2881 gagaaagcag aaagtgcacat tatgaacagg aagcctcggc acaagaaaaa ggacagactg
2941 gtgaaccagc agcttgctgt atactcgtac ctgcacattg gcctcatgca agccctggga
3001 gctttcctgg tgtacttcac tgtgtacgca cagcagggct ttgcggcgac ctactgttt
3061 cacctgcgga tagcgtggga cagcgaccac ctgaacgact tggaaagaaa ctatggacag
3121 gaatggacga gttatcagag gcaataacct gaatggacag gctacacggc tttctttgtt
3181 ggcatcatgg tccagcaaat agcagatctg atcatcagga agaccgcgaa gaactccatc
3241 ttcaagcagg ggctcttcag aaataaagt atctgggtgg ggtatcgctc ccagatcctc
3301 gtcgccctgc tcctctctta cgggctcggc agtatcacag ccttaaat t caccatgctc
3361 aaggctcagt actgggttgt ggccgtacct cagcccatcc tgatctgggt atacgatgaa
3421 atgcggaaac tcttcacatc gctctacccc ggaagctggt gggataagaa catgtattac
3481 tgagaccagg tctgtctctg agtctccag cggcacctgc ctgggtggtc tcggcaagac
3541 ctctgtgtag tgtggatggt gccaaagctc actcgggagg agactctcat ctagaacaca
3601 gtgggtgaagc ttcttactga tctgtgtac ttcaaagctg agattcagct gtttgtatat
3661 gattttctatc tctatctcca tctccttacc ttaaaagatg tggatgtcaa ggtcatggtg
3721 tagggaagga tgtgtttatc tgtatatgaa gctcactgat gtcacacaga cttgtgtaac
3781 ccaggtggct gctggagtct gccataagtt gagctagaat tgctcagatc tccttcacaa
3841 cctgtcaaaa ggcccggtga gctccatagg atttctgtga atccccctga aacataaactt
3901 ttgggggttg ctctgtctcag ctgagggtgt gagtgggaag tgtggcagca ggagcacctc
3961 agaacagcaa agacagcccc cgttttgact ccagacact ttgttctgtg gatgggttcc
4021 tggccatgcy gccccagttc gcctctctac agcactccac cactgtgtcc tgcaagctg
4081 acctccaagt ccattccaca aaccttaact caaacattcg tggacccaaa ggggtgtgca
4141 ctgactggga ctccggcctct ccggaagacc actgtgggtt agatagcact atttatttct
4201 ttagatagag ctgccaagca ctctccagca gccattttat gtcaatcaca tttttgtaac
4261 ttagatata tttgtgtgga caccgaaacac atacatccat gttgacaggt tttttttttt
4321 aataaaaaga tgtttttaag taaaatgttt tatgaaacaa aatctaattg tgatgtttta
4381 cttaattcaa gtttttccag aggcaggcac ggaataatcc aa

```

//

Figure 4-5--continued

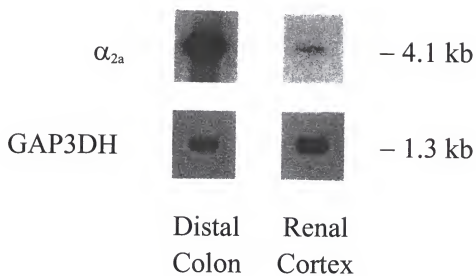


Figure 4-6. Northern analysis showing presence of HK α_{2a} in distal colon and renal cortex. GAP3DH was used to show the condition of the RNA samples.

To determine the relationship between the rabbit HK α_2 mRNAs and other P-type ATPases, phylogenetic analysis was necessary. Several programs and algorithms were sampled, all giving the same general pattern of sequence relationships. Programs employed include CLUSTALW (Thompson *et al.*, 1994), DISTANCES (Genetics Computer Group, 1997), and PAUP (Genetics Computer Group, 1997). All three programs use the distance algorithm, and the maximum parsimony algorithm of PAUP was also used. In addition to distance and maximum parsimony analyses, a third popular algorithm to assay relatedness of sequences is used, the maximum likelihood analysis. Distance analysis generates a tree showing relatedness of sequences by simply counting dissimilarities in aligned sequences as a test of their homology. Maximum parsimony considers only positive information in making a comparison. For instance, in an alignment of four sequences, a position that had one each of A, C, G, and T would not be included in the analysis because that position would not give information about any pair of the sequences being related. A position that had two As and two Cs would be counted because it would show the grouping of two pairs. Distance analysis would include both positions in the analysis. Maximum likelihood takes into account the tendency for a given type of mutation to occur. In two sequences that have a point mutation, that point mutation was more probably created by a "likely" change than a change that has less tendency to occur. Maximum likelihood would measure two sequences as more related if their alignment shows a "likely" mutation. If the mutation had less tendency, it may indicate a more distant relationship between sequences arrived at only by multiple changes

or higher pressure of selection. The maximum likelihood analysis requires information that is not readily available for rabbit, and was not attempted. A representative phylogram arrived at by distance analysis by the CLUSTALW program is shown in Figure 4-7.

The 5' end of the second rabbit sequence (HK α_{2c}) differed dramatically from the published rat HK α_{2a} (Crowson and Shull, 1992) and human HK α_{2a} (Grishin *et al.*, 1994) sequences. The HK α_{2c} 5' untranslated region bore no homology to the comparable segments from the HK α_{2a} cDNAs from human or rat, or to any GenBank sequence. It was clear from the beginning that this second 5' end might represent an alternative splicing product; the sequence homology diverges from the human HK α_2 sequence at a point known in the human ATP1A1 gene to be a splice junction (nucleotide 177 of ATP1A1) (Sverdlov *et al.*, 1996).

Alternative Splicing of H,K-ATPase α Subunits in the Kidney

When a sequence for the 5' end of the rat HK α_2 gene was published (Kone and Higham, 1998), we wanted to compare the sequence at the 5' end of the rabbit gene. In order to determine the exon arrangement at the 5' end of the HK α_2 gene, rabbit genomic DNA was amplified by PCR. The antisense primer was selected within the region of sequence that is common to HK α_{2a} and HK α_{2c} . Because it was unknown which of the two cDNA 5' ends was located more 5' in the gene, sense primers that anneal to sequence within each were selected. The oligonucleotide primer specific to HK α_{2a} gave the larger amplified product, and its sequence is shown in Figure 4-8A. The exon specific to HK α_{2a} splices to the common core sequence at the splice site shown. The exon specific to HK α_{2c}

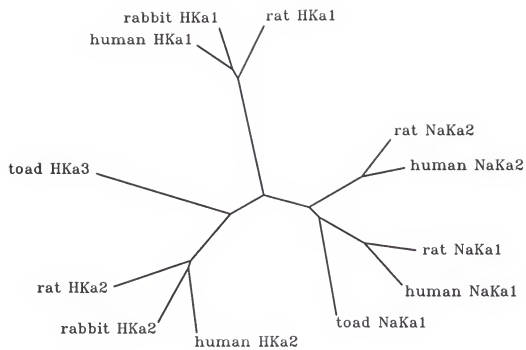


Figure 4-7. Distance analysis of selected HK α and NaK α subunit coding.

is continuous with the core sequence and lies within the first intron of the HK α_{2a} pre-mRNA. This general intron/exon structure is the same as that reported for rat HK α_{2a} and HK α_{2b} (Kone and Higham, 1998).

Expression of H,K-ATPase α Subunits in the Kidney

The start codon of HK α_{2a} was omitted in the HK α_{2c} sequence. Instead, a probable start codon was located upstream in frame with the HK α_{2a} open reading frame. Thus, the deduced amino acid sequence encoded a protein 61 amino acids longer at the amino terminal end than the rabbit HK α_{2a} sequence. The deduced amino acid sequence of the HK α_{2a} and HK α_{2c} proteins are shown in Figure 4-8A, indicating translational start sites. Although the HK α_{2c} cDNA indicated a continuous open reading frame including the amino-terminal extension, the possibility remained that translation might be initiated at the ATG codon homologous to that reported for the rat (Kone and Higham, 1998). Therefore, to determine whether the upstream ATG served as a translational start site, antipeptide antibodies were generated for peptides corresponding to amino acids 13-25 and 79-98 of the HK α_{2c} subunit. The former (antibody LLC27) was HK α_{2c} -specific, while the latter (antibody LLC25) recognized a segment common to both HK α_{2a} and HK α_{2c} subunits. Western analyses of rabbit kidney tissues and RCCT-28A cells using antibody LLC25 (anti-HK α_2 common) revealed a doublet migrating at an apparent molecular mass of approximately 90KDa (figure 4-9A). Experiments using antibody LLC27 (anti-HK α_{2c}) indicated a single band with a migration comparable to the upper band of the doublet (figure 4-9B). Both antibodies appear to recognize the same protein providing strong

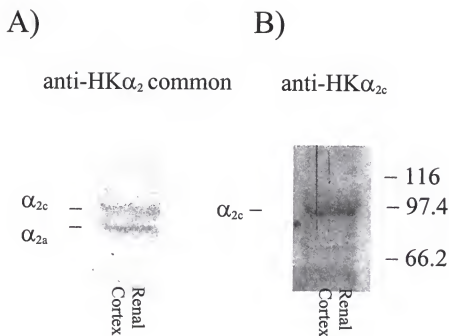


Figure 4-9. Western analysis showing presence of HK α_a and HK α_c protein in renal cortex. A) Detected with the anti-HK α_2 common antibody LLC25. B) Detected with the anti-HK α_{2c} -specific antibody LLC27. A and B show separate lanes from the same SDS-PAGE gel. Membrane was cut after electrotransfer and re-aligned after autoradiography.

evidence that the $\text{HK}\alpha_{2c}$ subunit containing the amino-terminal extension was indeed present in both rabbit renal tissue and RCCT-28A cells.

To test the hypothesis that enzyme localization in the kidney or in the cell is changed as a result of the alternative splicing of the mRNA, immunohistochemistry experiments were performed. Using our antibodies, Dr. Jill Verlander and Ms. Robin Moudy conducted immunohistochemistry experiments. Neither antibody we had made to the anti- $\text{HK}\alpha_2$ common region (LLC24 or LLC25) worked well for immunohistochemistry. However LLC22 (anti- $\text{HK}\alpha_1$) and LLC26 (anti- $\text{HK}\alpha_{2c}$) gave satisfactory results. A representative section photographed in Dr. Verlander's laboratory is shown in Figure 4-10. There is no visible reactivity except in the apical membranes of some cells of connecting segment and collecting duct. A majority of the cells in which labelling is observed bulge out into the lumen, a hallmark of the intercalated cell. Fewer cells are labelled in connecting segment compared to more distally in the collecting duct. This is in accordance with the normal distribution of acid-secreting intercalated cells, fewer in connecting segment than farther down in cortical collecting duct. There appears to be some labelling of cell types other than intercalated cells, including principal cells. Labelling continues to be visible in more distal sections of collecting duct than one would expect based on distribution of intercalated cells, but individual rabbits may vary widely in this distribution. Therefore, the few observations made do not permit a certainty that $\text{HK}\alpha_{2c}$ subunit protein is expressed more distally in cell types not normally thought to be associated with acid secretion. The lack of basolateral labelling implies that there is not a cell polarity change in $\text{HK}\alpha_{2c}$ protein compared to $\text{HK}\alpha_{2a}$.

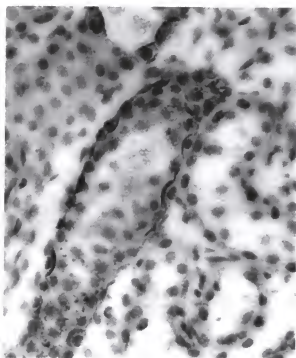


Figure 4-10. Immunohistochemistry by Dr. Jill Verlander and Ms. Robin Moudy. Anti-HK α_2 antibody reacts with the apical surfaces in some cells in rabbit renal collecting duct. The section shown is from the outer stripe of outer medulla.

Discussion

We have generated complete cDNAs for both the rabbit renal HK α_{2c} and the novel HK α_{2c} subunits. They were more closely related to other HK α_2 nucleotide sequences than to HK α_1 or to Na,K-ATPases. The exon structure at the 5' end was found and compared to the exon structure found at the 5' end of rat (Kone and Higham, 1998). Proteins corresponding to both HK α_2 isoforms were detected by immunoblot analysis, indicating that the novel HK α_{2c} has the predicted amino-terminal extension.

The HK α_2 cDNAs reported here generated using rabbit renal cortex RNA as template have high homology to previously known HK α_2 sequences from human skin axilla and the rat distal colon (Grishin *et al.*, 1994; Crowson and Shull, 1992). The level of homology is relatively low compared to the homology typically found when comparing HK α_1 or NaK α subunits across mammalian species. However, it is much higher than the homology between the rat HK α_1 and HK α_2 cDNAs (Crowson and Shull, 1992) or among the different Na,K-ATPase isoforms within a species. The HK α_2 isoforms appear to have undergone somewhat greater evolutionary divergence than HK α_1 or the Na,K-ATPase catalytic isoforms. Phylogenetic analysis provides a clearer picture of the relationships between these P-type ATPases. In a phylogram (figure 4-7), the HK α_2 subunit isoforms cluster together compared to the other P-type ATPases. The HK α_2 subunit nucleotide sequences all represent H,K-ATPases that are expressed at high level in colon, at lower level in other tissues including the kidney, and not at all in stomach. At least in rat and

rabbit, an alternatively spliced variant has been shown to be transcriptionally competent, and the pattern of introns and exons that give rise to this alternative splicing are strikingly similar. It would be highly interesting to attempt to detect a similar transcript in human. Based on tissue distribution, phylogenetic analysis, and the similarity of alternatively spliced transcripts, it continues to be a valid premise that the HK α_2 subunits known for rat, human, and rabbit form an orthologous group.

Like the rat, the rabbit appears to have alternatively spliced transcripts of HK α_2 in the kidney. The organization of the rat alternatively spliced cDNA (Kone and Higham, 1998) omitted the exon containing the start codon of the sequence previously reported for rat distal colon (Crowson and Shull, 1992), giving rise to a protein truncated by 108 amino acids at the amino-terminal end. The rabbit HK α_{2c} sequence also omits the start codon present in the HK α_{2a} sequence. However, an upstream 5' ATG codon lies in the same uninterrupted reading frame as the coding sequence for HK α_2 . Initiation of translation at this position yields a subunit having an extended amino-terminus 61 amino acids longer than the canonical HK α_{2a} protein. Western analysis demonstrated that a protein having this extension is present in rabbit kidney. An antibody designed to detect the common core region of HK α_2 was reactive to two proteins very close in apparent mass, whereas an antibody designed to detect the amino-terminal extension was reactive to a single species.

The extended portion of the HK α_{2c} -encoded protein contains a casein kinase II phosphorylation motif at *thr-12*, and a cAMP-dependent protein kinase phosphorylation motif at *thr-53*. These sites impart a potential for regulation of HK α_{2c} distinct from HK α_{2a} . There are no apparent glycosylation sites or signal sequences. The HK α_{2c} extension is

hydrophilic in nature and lacks any conspicuous membrane-spanning domains. Because the N-terminus of HK α_1 was found to be cytosolic (Smolka *et al.*, 1992), the elongation can be expected to have a cytosolic location. Figure 1-1 shows the putative location of the amino-terminal extension. Chou-Fasman calculations predict this segment to be predominantly alpha- helical with a turn structure in a region containing seven prolines between amino acids 26 through 40. A similarly proline-rich hinge region is found in band 3 protein, and an ankyrin-binding site has been localized to that area of band 3 (Willardson *et al.*, 1989). Products of alternative splicing of the band 3 protein AE1 gene have been characterized in chicken kidney, where it is thought that the variation in transcripts serves to determine the membrane domain to which the polypeptides are targeted (Cox *et al.*, 1995). A similar situation may exist for the various HK α_2 transcripts; alternative splicing may mediate the polarity of expression. In cortical thick ascending limb, an H,K-ATPase activity has been described that diminished in rats fed a low K⁺ diet (Younes-Ibrahim *et al.*, 1995). Basolateral polarity of expression would be consistent with potassium homeostasis in that case. However, no basolateral staining using the anti-HK α_{2c} antibody was seen in this segment of the nephron (discussed below). If the HK α_{2b} protein is the molecule responsible for that activity, its absence in RCCT-28A could be explained by the normal expression being in a region of the cortex other than CCD.

A number of possibilities exist to answer the question of why there are multiple H,K-ATPases in the kidney. One possibility is differential regulation. In rat (Kone and Higham, 1998) and rabbit (this work) the alternatively spliced HK α_2 subunit mRNA contains multiple upstream open reading frames. This may be associated with an inhibition of translation, leading to a decrease in HK α_2 subunit protein in the cell. Covalent

modification is another possible means of differential regulation. In this case, phosphorylation is an unlikely candidate for differential regulation because although motifs recognised by kinases are present, they are not conserved between rat (Kone and Higham, 1998) and rabbit. Having a different promoter region immediately 5' to the HK α_{2c} cDNA gives rise to potential differences in regulation relative to HK α_{2a} . Of course, different promoter regions giving different responses to conditions such as aldosterone status and low K⁺ may account for why there are the three catalytic subunit isoforms HK α_1 , HK α_{2a} , and HK α_{2c} . There may also be differences in the kinetics between HK α_{2a} and HK α_{2c} .

Another possibility is localization, both within the kidney and within the cell. Using our antibodies, Dr. Jill Verlander and Ms. Robin Moudy conducted immunohistochemistry experiments. They found a similar pattern of expression of expression of HK α_{2c} compared to other H,K-ATPase proteins for which localization is known (Wingo *et al.*, 1990; Campbell-Thompson *et al.*, 1995; Ahn and Kone, 1995; Ahn *et al.*, 1996; Haragsim and Bastani, 1996). These results imply that the alternative splicing does not confer on the protein a different polarity of expression or localization within the kidney. They do imply that the HK α_{2c} protein joins other acid and ion transporters in cell types specialized for high transport activity.

Immunohistochemistry showed that differences in HK α_{2c} subunit protein cellular distribution had no major departures from the distribution of other H,K-ATPase subunits. Because the phosphorylation motifs in rat and rabbit alternatively spliced isoforms are not conserved, regulation by phosphorylation is a less likely candidate to lead to differential regulation. There are myriad possibilities for why there is such a heterogeneity of

H,K-ATPases in the kidney, such as differential regulation at the gene level by different promoter regions, differential regulation of protein synthesis by the short upstream open reading frames, or differences in enzyme kinetics.

H,K-ATPASES IN A RABBIT KIDNEY CORTICAL COLLECTING TUBULE A-TYPE INTERCALATED CELL LINE

Introduction

The kidney is a well-organized and complicated organ, with many different cell types contributing to its morphology and function. Any understanding of the kidney must include an appreciation of the processes mediated by each cell type. Expression of H,K-ATPase has been shown to be complex in rabbit kidney, so one issue of interest is the identification of the cell types possessing the various isoforms of the pump.

Immunohistochemical evidence was presented in the previous chapter indicating that the collecting duct A-type intercalated cell is one of the primary cell types that expresses H,K-ATPase catalytic subunit proteins. An independent approach showing that A-type intercalated cells express these pumps is to detect H,K-ATPase in a well-characterized cell line of intercalated cell origin. A cell line in which H,K-ATPase was present would also serve as a model cell system offering the potential for future experiments pertaining to renal H,K-ATPases.

The rabbit cortical collecting tubule cell line RCCT-28A was selected by immunodissection (Arend *et al.*, 1989). Cortical collecting duct was dissected under a microscope and the collagenase-dispersed cells incubated in culture dishes to which the antibody rct-30 was attached (Spielman *et al.*, 1986). The rct-30 antibody binds specifically to collecting duct cells. The resultant cell line was shown to have

characteristics of the cortical collecting duct A-type intercalated cell (Arend *et al.*, 1989; Schwiebert *et al.*, 1992; Dietl *et al.*, 1989; Bello-Reuss, 1993). The existence of H,K-ATPase mRNA, protein, and activity in this cell line was studied to demonstrate that this is a cell type that expresses H,K-ATPase. Here we show by RT-PCR that mRNA for HK α_1 , HK α_{2a} , HK α_{2c} , and HK β are present in RCCT-28A cells. By western analysis it is shown that protein corresponding to the novel alternatively spliced isoform HK α_{2c} is present. Lastly, fluorescence microscopy measurements are used to demonstrate that RCCT-28A cells have a mechanism for pH_i regulation that is K⁺-dependent and sensitive to the H,K-ATPase inhibitor Sch-28080. Together the data represent very strong evidence for H,K-ATPase expression in a cell line derived from the type-A intercalated cell in the collecting duct.

Detection of H,K-ATPase in RCCT-28A Cells

Detection of H,K-ATPase mRNAs in RCCT-28A cells

To determine the presence of H,K-ATPase transcripts, we examined whether the RCCT-28A cells possessed mRNA for the known H,K-ATPase β or α subunits. In the initial experiments, there were no H,K-ATPase subunits detectable by northern analysis, nor were there RT-PCR products amplified from RCCT-28A cells that could be directly visualized by agarose gel electrophoresis. A more sensitive technique, Southern blotting of the RT-PCR products using cDNA probes designed to hybridize to the expected products was employed to demonstrate the presence of H,K-ATPase subunit mRNA in these cells.

RT-PCR products were amplified using RCCT-28A cell total RNA as a template. The presence of HK β subunit mRNA was observed as a product of approximately 309 bp hybridizing to a probe containing nucleotides 304-873 of the rabbit HK β sequence obtained by Reuben *et al.* (1990) in gastric tissues (Figure 5-1A). Sizes were estimated by measurements from the original agarose gel on which a 100 bp ladder was visualized by ethidium bromide staining. The "-RT" lanes in this and all subsequent figures depict negative controls in which RT was omitted from reactions to show that the RNA template was free of contaminants. Products amplified from mRNA isolated from rabbit renal cortex tissue were also included in these experiments as a positive control for the RT-PCR reaction. The hybridization of a probe specific for HK β to an RT-PCR product of the expected size implied the presence of HK β in these cells.

At the time these experiments were carried out, the existence of alternatively spliced HK α_2 variants had not yet been discovered. Therefore, these experiments were designed only to show the presence of HK α_2 transcript in general, and do not differentiate between the HK α_{2a} and HK α_{2c} species. For HK α_2 mRNA, a primer pair yielded the anticipated product size of 305 bp. This product was hybridized with a probe of nucleotides 1264-1569 of the HK α_{2a} sequence, contained in the region now known to be common to HK α_{2a} and HK α_{2c} (Figure 5-1B).

A similar strategy was employed to show that HK α_1 was present in the RCCT-28A cells. Primers were designed to amplify a 611 bp region of HK α_1 mRNA (nucleotides 2537-3147), and again a product of the expected size was observed to hybridize to a probe containing the same 611 bp region (Figure 5-1C). At the time these experiments

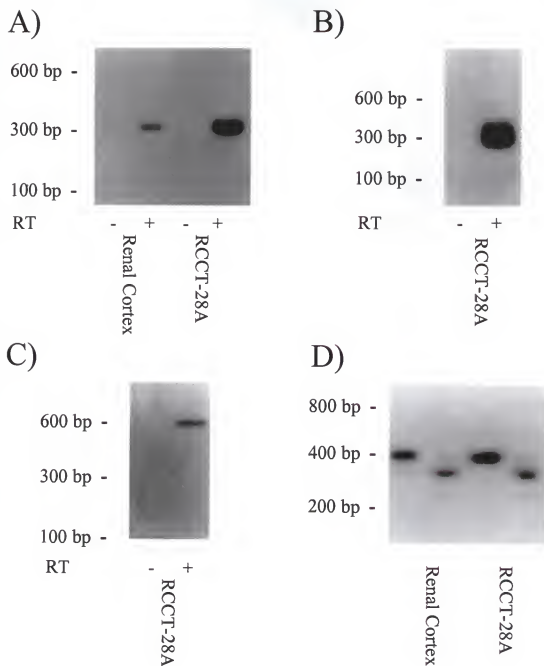


Figure 5-1. Southern blots of H,K-ATPase subunit mRNA in RCCT-28A cells. A) Products amplified by RT-PCR were shown by Southern blot analysis. PCR primers were designed to amplify a 309 bp region within the 3' UTR of H,K-ATPase β subunit. B) PCR primers designed to amplify a 305 bp region within the coding sequence of H,K-ATPase α_2 subunit produced the products shown. C) PCR primers designed to amplify a 611 bp region within the 3' UTR of H,K-ATPase α_1 subunit produced the products shown. D) Restriction digests of the 611 bp fragment of H,K-ATPase α_1 . *Bam* HI digestion was predicted to yield fragments of 423 and 187 bp. *Pst* I digestion was predicted to yield fragments of 353 and 257 bp.

were ongoing, there were no reports in the literature of HK α_1 in kidney, so a further step was taken to confirm the identity of these products. Restriction digests were carried out on these products, and the anticipated size fragments were obtained (Figure 5-1D), further evidence that these products corresponded to HK α_1 mRNA in RCCT-28A cells.

With further optimization of RT-PCR protocols, including such parameters as annealing temperatures and number of amplification cycles, it was found that the RT-PCR technique was capable of generating products visible by ethidium bromide staining of agarose gels. This was a substantial improvement over the preceding experiments, because it offered the opportunity to obtain nucleotide sequences of the PCR products to confirm their identity. RT-PCR products were generated and sequenced using RCCT-28A cell total RNA as a template. The presence of HK β subunit mRNA was observed by amplification of a 570 bp cDNA corresponding to nucleotides 304-873 of the sequence obtained by Reuben *et al.* (1990) in gastric tissues (Figure 5-2A). Nucleotide sequencing of the RCCT-28A product confirmed that the amplified product was identical with the rabbit HK β subunit mRNA.

HK α_1 was also shown to be present in the RCCT-28A cells by the same technique. Primers were designed to amplify a 611 bp region of HK α_1 mRNA (nucleotides 2537-3147), and again a product of the expected size was observed (Figure 5-2B). The nucleotide sequence was identical to that reported by Bamberg *et al.* (1992) except for two single base mismatches. One mismatch was a transition of G $\rightarrow$ A at nucleotide 2567 and the other a G $\rightarrow$ C transversion at nucleotide 3089; neither affected the deduced amino acid sequence.

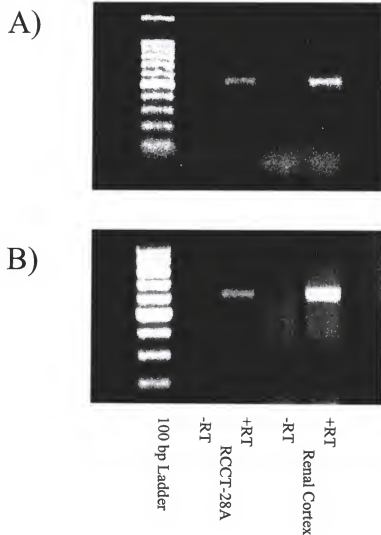


Figure 5-2. HK β and HK α , subunit mRNA in RCCT-28A cells. PCR products were generated using RCCT-28A cell and rabbit renal cortex total RNA. Amplification was 40 cycles for RCCT-28A cell PCR products and 30 cycles for renal cortex PCR products. 100 bp ladder is shown for size reference.

For HK α_2 mRNA, a primer pair yielded the anticipated product of 305 bp, which was identical to nucleotides 1264-1569 of the HK α_{2a} sequence. This segment was contained in the region common to HK α_{2a} and HK α_{2c} (Figure 5-3A). To determine whether HK α_{2a} and HK α_{2c} isoforms were both present in RCCT-28A cells, primer pairs were designed that amplify regions specific to each isoform. HK α_{2a} -specific primers amplify an 86 bp (nucleotides 7-93 in the HK α_{2a} sequence) RT-PCR product from RCCT-28A cellular RNA and nucleotide sequence determination showed the product to be identical to the sequence of the HK α_{2a} cDNA from rabbit cortex (Figure 5-3B). A product of 354 bp (nucleotides 24-377 in the HK α_{2c} sequence) amplified using rabbit renal cortex mRNA as template was also generated (Figure 5-3C). The sequence of this product was identical to the HK α_{2c} cDNA sequence from rabbit renal cortex. Therefore, RCCT-28A cells contained the mRNAs encoding all three HK α subunits and the HK β subunit. Since RCCT-28A cells are a clonal cell line, the implication is that individual A-type intercalated collecting duct cells may express at least three different H,K-ATPases.

Detection of HK α subunit protein in RCCT-28A cells

With convincing evidence of the presence of mRNA for these H,K-ATPase subunits in hand, the next logical experiments was to demonstrate that RCCT-28A cells also produced H,K-ATPase proteins. We employed the antibodies described in previous chapters to determine the H,K-ATPase catalytic subunits present in these cells. Using western analysis and one of the antibodies to the common core of the HK α_2 proteins (LLC25 anti-HK α_2 common) and two of the antibodies specific to HK α_{2c} (LLC26 and

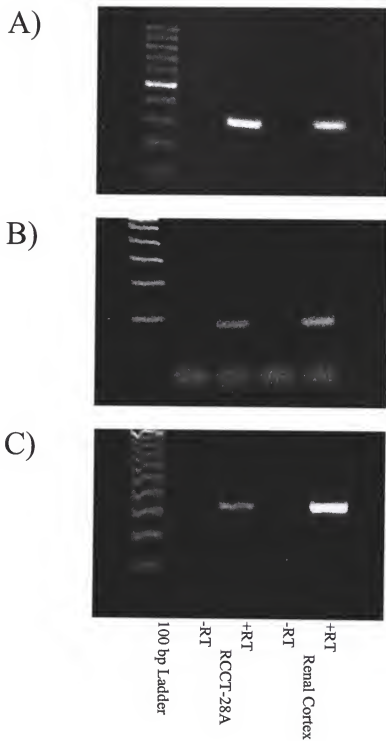


Figure 5-3. HK α subunit mRNA in RCCT-28A cells. PCR products were generated using RCCT-28A cell and rabbit renal cortex total RNA. Amplification was 40 cycles for RCCT-28A cell PCR products and 30 cycles for renal cortex PCR products. 100 bp ladder is shown for size reference.

LLC27 anti-HK α_{2c}), it was demonstrated that at least the HK α_{2c} protein was present in these cells. The second antibody to the common core protein (LLC24 anti-HK α_2 common) failed to show reactivity in the RCCT-28A cells in initial experiments, and all further western analysis was done using LLC25 (anti-HK α_2 common). Of the two HK α_{2c} -specific antibodies, LLC27 (anti-HK α_{2c}) gave the least background so it was selected for all further western analysis.

In Figure 5-4A, immunoreactivity of LLC25 (anti-HK α_2 common) to membrane proteins of RCCT-28A cells is shown. Reactive protein was detected migrating at an approximate molecular mass of 90 kDa, consistent with the presence of HK α_2 subunit protein in these cells. Although this broad band may represent a doublet, this has not been clearly resolved. Figure 5-4B shows the results of western analysis of RCCT-28A protein and the LLC27 (anti-HK α_{2c}) antibody. A band was visible corresponding to a molecular mass of 90 kDa, consistent with the presence of HK α_{2c} subunit protein in the RCCT-28A cells. Based on these data one may conclude that an HK α_{2c} subunit protein was present, but because two species were not discernable using LLC25 (anti-HK α_2 common) it was not proven that HK α_{2a} subunit protein was there as well. Given the results using LLC25 (anti-HK α_2 common) and LLC27 (anti-HK α_{2c}) antibodies, it might be argued that only HK α_{2c} was present in RCCT-28A cells. It was only with difficulty that the doublet indicative of both HK α_2 proteins were observed in kidney, so not observing a doublet in the RCCT-28A cell western should not be taken as proof of the absence of HK α_{2a} protein. Importantly, the preceding mRNA detection experiments showed that both alternatively spliced transcripts were present in these cells.

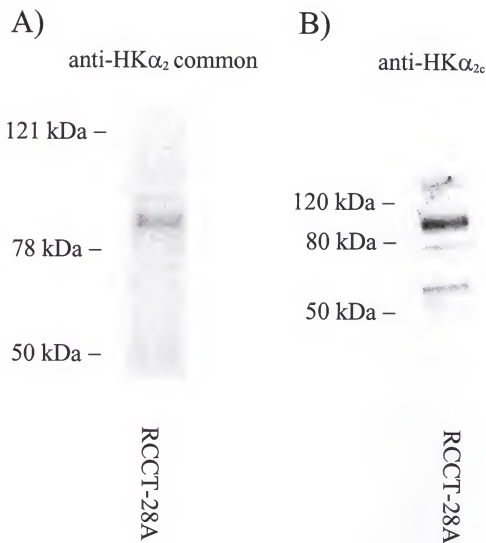


Figure 5-4. Western analysis showing presence of HK α_2 and HK α_{2c} protein in RCCT-28A cells. A) Detected with the anti-HK α_2 common antibody LLC25. B) Detected with the anti-HK α_{2c} -specific antibody LLC27.

In preliminary experiments involving RCCT-28A cells, the HK α_1 -specific antibodies we produced (LLC22 and LLC23 anti-HK α_1) did not show reactivity. However, using RCCT-28A cells we provided, Dr. Adam Smolka of the Medical University of South Carolina did show reactivity to the HK α_1 antibody HK12.18 (personal communication).

Detection of H,K-ATPase activity in RCCT-28A cells

H,K-ATPase subunits were not detected in RCCT-28A cells by northern analysis, and the subunits were apparently at relatively low abundance by western analysis. These results implied that H,K-ATPase expression was at a low level in these tissue culture cells. Because mRNA and protein levels were so low, it was necessary to determine a basal H,K-ATPase activity in these cells. To confirm by an independent method that the RCCT-28A clonal cell line has H,K-ATPase activity as previously reported (Bello-Reuss, 1993), fluorescence microscopy with digital video image analysis was used to measure pH_i recovery rates of individual cells after an acid load.

These experiments were undertaken in the laboratory of Dr. David Weiner, who had a decade of experience making similar measurements, and kindly provided his equipment and expertise. The development of a technique for measuring H,K-ATPase activity was important for future studies undertaken by our lab, and the technique developed here offered advantages over those previously used. This technique measures activity of individual cells in order to achieve maximal sensitivity and to assess the homogeneity of the population of tissue culture cells. Additionally, this technique used the same apparatus used to measure H,K-ATPase activity in perfused tubules, allowing comparison to activities measured in a system more representative of the kidney in the whole animal. The

actual protocols used underwent some evolution as described below. Results are first described for the early protocols used to provide an appreciation for how the differing protocols affected the measurements. Final results obtained by the refined protocol then follow.

The technique used to measure H,K-ATPase activity in this work consisted of fluorescence microscopy to observe changes in pH_i of single cells while cells were perfused by solutions containing various substrates and inhibitors. For example, H,K-ATPase activity requires K^+ entry for intracellular alkalinization. If H,K-ATPase activity were the only method of acid extrusion available to an acidified cell, pH_i would be expected to rise back to its equilibrium pH_i in solutions containing K^+ due to exchange of extracellular K^+ for intracellular H^+ . In solutions lacking K^+ , this exchange would be absent, and cells could not recover. The rate of pH_i recovery (pH units/min) in the presence of K^+ would be proportional to H,K-ATPase activity. Another maneuver that was used to identify H,K-ATPase activity was to measure recovery rates in the presence and absence of an H,K-ATPase inhibitor. Like the absence of K^+ , the presence of the inhibitor would block recovery of an acidified cell to normal pH_i . By inhibition of other mechanisms of pH_i regulation, H,K-ATPase activity can indeed be isolated and rates determined in this way.

pH_i was determined by changes in fluorescence of the pH-sensitive dye BCECF. The acetoxymethyl ester of BCECF crosses cell membranes freely, and intracellular esterases cleave the ester bond trapping BCECF within the cell. Therefore, BCECF-AM was the agent used to load cells with BCECF for pH_i measurements. After BCECF loading, cells were transferred to the microscope stage. The next step of the experiment was acid

loading of the cells. This was done using an NH_4Cl prepulse. *Solution 2* (Table 2-2) contained the neutral, membrane-permeant ammonia which entered the cell and equilibrated to produce an amount of the weak acid ammonium. Upon removal of ammonia from the extracellular solution, intracellular ammonia escaped the cell down its electrochemical gradient. Ammonium was trapped within the cell by its membrane impermeance, the electronegativity of the cell interior, and the fact that NH_4^+ is transported inwardly by Na,K-ATPase . This trapped ammonium acidified the cells. As already noted, following this acidification, measurements may be made of transporters that mediate acid extrusion and thus give rise to alkalization of the cell. Following these measurements, the Na^+/H^+ exchanger was released from inhibition, and in functional cells a robust recovery toward equilibrium pH_i for the cell ensues.

Once pH_i for all the cells in the field of view of the microscope was recorded by the computer for each time point in the experiment, analysis of the individual cells may begin. The FL/1 program allows the selection of single cells, and the pH_i is plotted through the different changes of solution. Rates of change in pH_i for the relevant periods may be calculated by a spreadsheet computer program. It is these rates of recovery that were the basis of defining H,K-ATPase activity.

The most robust and ubiquitous mechanism of pH_i regulation in cells is the Na^+/H^+ exchanger. The relatively large contribution to recovery from an acid load mediated by the Na^+/H^+ exchanger obscured recovery by other mechanisms, so its activity had to be blocked for measurements of activities of other H^+ transporters. In the initial studies of this work, Na^+ removal was used to block Na^+/H^+ exchanger activity. In these studies, described more fully below, H,K-ATPase activity pH_i recovery rates were low. At the

time, Dr. Weiner was attempting without success to detect H,K-ATPase activity in A-type intercalated cells in perfused tubules. A suggestion by a reviewer of a manuscript from Dr. Weiner's laboratory was to utilize the potent amiloride analog EIPA to inhibit the Na^+/H^+ exchanger instead of using removal of Na^+ . As seen below, measurements of H,K-ATPase-mediated recovery roughly doubled in the presence of Na^+ with EIPA, and this must be regarded as a major improvement of this assay compared to its original form. Hays and Alpern (1991) had previously observed an effect of Na^+ removal on pH_i regulation by H^+ pumps, and in its final form, the study of A-type intercalated cells by Milton and Weiner (1997) observed a similar effect.

Another major evolution of these studies was the finding that there were time-dependent effects that appear after acid loading. These were never observed in control experiments at a time less than 8 min following NH_4Cl removal. After the 8 min period, two separate effects that would disturb rate measurements were seen in some cells. The first was that in some cells the recovery would plateau before attaining the normal equilibrium, baseline pH_i . When Na^+ was returned to solutions, or when EIPA was removed, functional cells fully recovered toward baseline. This plateau effect has been observed in previous studies of Na^+ -independent pH_i regulation (Montrose and Murer, 1986, Montrose *et al.*, 1987). The second confounding effect was that in the continued absence of potassium a delayed increase in the pH_i recovery rate developed, consistent with a delayed stimulation of H^+ -ATPase. These results were consistent with previous results by Hays and Alpern (1991), who described an H^+ -ATPase activity that was delayed following an NH_4Cl prepulse. Bello-Reuss (1993) also detected H^+ -ATPase as well as H,K-ATPase activity in RCCT-28A cells. As a consequence, the original protocols calling

for a period of K^+ absence followed by a period of K^+ addition had to be modified. Experiments involving perfused tubules typically involved taking data in absence then presence of solution substrates or inhibitors, but in this system there was not sufficient time following acid loading for rates to be accurately determined for both K^+ absence and K^+ presence. Therefore, the final design of the protocol called for making multiple separate measurements of K^+ -dependent recovery rates and K^+ -independent recovery rates. Sch-28080-sensitive recovery rates were also determined in multiple separate experiments. With measurements made in this way, there was a statistically significant difference between the experimental conditions with the measurements being consistent with the presence of an H,K-ATPase in RCCT-28A cells.

Apical K^+ -dependent RCCT-28A cell pH_i regulation in the absence of Na^+ . Figure 5-5 shows a representative tracing of a cell that was acid loaded by an NH_4Cl prepulse (solution 2 added to basolateral chamber) and pH_i recovery observed for a period of 5 min in the absence of Na^+ , HCO_3^- , or apical K^+ (solution 3 in apical chamber, solution 4 in basolateral chamber). Under these conditions, recovery of pH_i to physiological range mediated by such processes as Na^+/H^+ exchange, Cl^-/HCO_3^- exchange, or apical H^+/K^+ exchange would be blocked. A population of cells was found in which the mean recovery pH_i rate was 0.000 ± 0.001 pH units/min under these conditions, not significantly different from zero. Upon switching the media perfusing the apical side to solution 4, a K^+ -containing solution, a mean recovery rate of 0.015 ± 0.004 pH units/min was observed, consistent with an apically located K^+ -dependent mechanism of pH_i recovery. With Na^+ added to the perfusing solution, a robust recovery of pH_i toward baseline value was observed, presumably mediated by Na^+/H^+ exchange.

NH_4^+	-	+	-
Na^+	+	-	+
Ap K^+	+	-	+
Bl K^+	+		

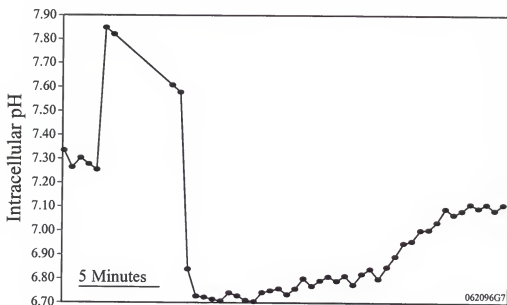


Figure 5-5. pH_i recovery from an acid load by RCCT-28A cells in the absence of Na^+ . A representative tracing of pH_i in an individual cell is shown. Periods during which solutions contain 20 mM NH_4Cl , 120 mM Na^+ , and 5 mM K^+ on apical (Ap) side are indicated. Throughout the experiment 5 mM K^+ was present on basolateral (Bl) side. Cells were acid loaded and recovery was observed in the absence of Ap K^+ , then in the presence of Ap K^+ . No K^+ -independent recovery was observed, but in the presence of K^+ there was detectable recovery. Finally, Na^+ was added back and robust recovery was observed.

K⁺-dependent RCCT-28A cell pH_i regulation in the presence of Na⁺. Because of the rather modest level of H,K-ATPase activity detected in these cells, and because of results obtained by Milton and Weiner (1997) indicating that Na⁺ must be present for optimal measurements of H,K-ATPase activity by this technique, we undertook studies in which Na⁺ was present during the pH_i recovery period following an acid load. The cells were acid loaded by symmetrical addition of *solution 5* containing 1 μ M EIPA. In the absence of K⁺ (symmetrical *solution 6*), a recovery rate that was not significantly different from zero (0.001 ± 0.003 pH units/min) could be measured in three separate cell preparations. A K⁺-dependent recovery (symmetrical *solution 1* plus 1 μ M EIPA) could be observed (0.032 ± 0.006 pH units/min, $P < .0001$ vs K⁺-free perfusion). There was approximately a three-fold activation of K⁺-dependent recovery rate pH_i attributable to the presence of Na⁺ in the perfusing solutions.

K⁺-dependent, EIPA-sensitive RCCT-28A cell pH_i regulation. The following measurements represent the conclusive experiments, in which Na⁺ is present for maximal H,K-ATPase activity and measurements were made during periods in which there were no K⁺-independent artifacts. A representative tracing of a cell acid loaded by an NH₄Cl prepulse (symmetrical *solution 5* plus 1 μ M EIPA) and pH_i recovery observed is shown in Figure 5-6A. In a majority of RCCT-28A cells there was significant EIPA-insensitive pH_i recovery in K⁺-containing solutions (symmetrical *solution 1* plus 1 μ M EIPA). For 136 cells from six independent passages of RCCT-28A cells, the mean recovery rate was 0.022 ± 0.005 pH units/min. In the absence of K⁺ (symmetrical *solution 6* plus 1 μ M EIPA) during the period following acid load no immediate pH_i recovery was observed (Figure 5-6B). In five separate experiments (129 cells), the K⁺-independent pH_i recovery

NH ₄ ⁺	-	+	-
EIPA	-	+	

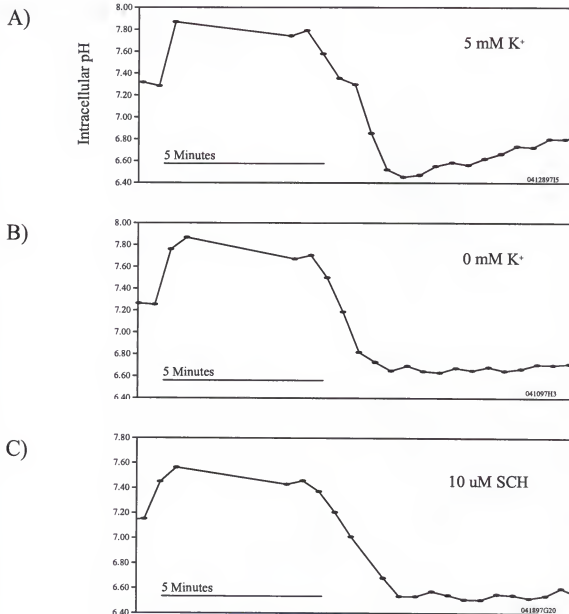


Figure 5-6. pH_i recovery from an acid load by RCCT-28A cells in the presence of EIPA. Representative tracings of pH_i in individual cells are shown. Periods during which solutions contain 20 mM NH₄Cl and 1 mM EIPA are indicated. Cells were acid loaded and recovery was observed in the presence of K⁺ (A), in the absence of K⁺ (B) and in the presence of Sch-28080 (C).

rate averaged 0.004 ± 0.002 pH units/min ($P < 0.01$ vs. 5 mM K^+). The K^+ -independent, EIPA-sensitive pH_i recovery rate measured immediately after acid loading was less than that measured during the protocols in which measurements were delayed with respect to acid loading.

Sch-28080- and EIPA-sensitive RCCT-28A cell pH_i regulation. To test the hypothesis that K^+ -dependent pH_i recovery from an acid load resulted from H,K-ATPase, the effect of the classical H,K-ATPase inhibitor Sch-28080 (10 μ M) was examined on RCCT-28A cell pH_i regulation (Figure 5-6C). After acid loading in *solution 7* containing Sch-28080 plus EIPA, cells were perfused in a K^+ -containing solution (*solution 6* with Sch-28080 plus EIPA). Using this protocol, 109 cells from four separate cell preparations had a mean recovery rate of 0.002 ± 0.002 pH units/min ($P < 0.05$ vs. 5 mM K^+ , $P = NS$ vs. 0 mM K^+) in the presence of 10 μ M Sch-28080.

Buffer capacity and pH_i after acid loading. The effects of the various protocols performed in these experiments might be explained by differences in buffer capacity or the degree to which cells are acidified by the NH_4Cl pre-pulse. Although these experiments directly measured the rate of pH_i change, the variable of interest is proton flux; these two values are directly related by buffer capacity. Thus, buffer capacity had to be considered to avoid misconceptions concerning proton flux changes based on observations of pH_i recovery rates. Buffer capacities measured by the NH_3/NH_4^+ technique (Boyarsky *et al.*, 1988) in the presence of K^+ , absence of K^+ , and presence of Sch-28080 were 15 ± 1 mEq H^+ /pH, U/L cell volume, 16 ± 1 ($P = NS$ vs. 5 mM K^+), and 16 ± 2 ($P = NS$ vs. 5 mM K^+), respectively. Therefore, changes in pH_i recovery rates among the protocols could not be attributed to changes in buffer capacities.

Some mechanisms of pH_i recovery have recovery rates that are influenced by absolute pH_i , with a tendency for greater acidification to stimulate higher rates of pH_i recovery. Therefore, a change in the rate of recovery might have been due to differences in absolute pH_i after acid loading. Actual nadir pH_i observed in these experiments in the case of K^+ presence, K^+ absence, and Sch-28080 presence measured 6.64 ± 0.06 , 6.59 ± 0.08 ($P = \text{NS}$ vs. 5 mM K^+), and 6.54 ± 0.03 ($P = \text{NS}$ vs. 5 mM K^+). Consequently, changes in pH_i recovery rates among the protocols was not due to the nadir pH_i .

Discussion

RCCT-28A cells contained the mRNAs encoding all three $\text{HK}\alpha$ subunits and the $\text{HK}\beta$ subunit. Since RCCT-28A cells are a clonal cell line, the implication is that individual A-type intercalated collecting duct cells may express at least three different H,K-ATPases. The $\text{HK}\alpha$ subunit mRNA detected in RCCT-28A cells gives rise to at least the $\text{HK}\alpha_1$ and $\text{HK}\alpha_{2c}$ proteins. It is of interest that the most novel subunit, the alternatively spliced $\text{HK}\alpha_{2c}$ protein is indeed present in the cortical collecting tubule A-type intercalated cell line, in agreement with the expectations brought by the immunohistochemistry studies. And like the immunohistochemical evidence both with LLC22 (anti- $\text{HK}\alpha_1$) and in previous work by others, the experiments with RCCT-28A cells adds to the evidence that $\text{HK}\alpha_1$ is also a transporter expressed in the collecting duct A-type intercalated cell.

We elected not to pursue detection of $\text{HK}\beta$, the putative partner to $\text{HK}\alpha_1$, due to time constraints and because of our interest in characterizing our own antibodies. It has quite recently (less than two months ago) been reported that $\text{NaK}\beta_1$ is a likely partner to $\text{HK}\alpha_{2c}$

(Dubose *et al.*, 1998; *et al.* Kraut *et al.*, 1998), so detection of that subunit has also not been done. The ubiquity of NaK β_1 in the kidney and in the whole animal would make a positive result by western analysis very difficult to interpret in terms of relevance to H,K-ATPase.

We have provided independent confirmation of Bello-Reuss' (Bello-Reuss, 1993) report that RCCT-28A cells have H,K-ATPase activity. Either the presence of the H,K-ATPase inhibitor Sch-28080 or the absence of K⁺ virtually abolished the intracellular alkalization rates of RCCT-28A cells following an acid loading (Figure 5-7). When K⁺ was present on only the basolateral side of RCCT-28A cells, mean recovery rates were not significantly different from zero. In the presence of K⁺, RCCT-28A cells possessed a mechanism for pH_i recovery that exceeded the rates observed without K⁺ or with Sch-28080. These results constituted an independent confirmation of apical H,K-ATPase activity in RCCT-28A cells.

Studies in heterologous expression systems (Codina *et al.*, 1996; Coughnon *et al.*, 1996; Lee *et al.*, 1995; Modyanov *et al.*, 1995) and in a mammalian expression system (Grishin *et al.*, 1996) suggested that an H,K-ATPase with the HK α_2 subunit was less sensitive to Sch-28080 inhibition than the HK α_1 pump, thus the differential sensitivity of the two enzymes offered the possibility of discriminating between the two at the level of activity. In this study the K⁺-independent and Sch-28080-sensitive pH_i recoveries were similar, arguing that HK α_1 is the isoform responsible for basal levels of H,K-ATPase activity in RCCT-28A cells. Buffin-Meyer *et al.* (1997) observed a like situation in rat

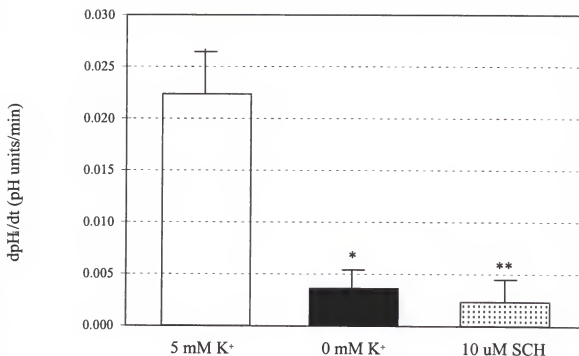


Figure 5-7. Summary of the rates of pH_i recovery from an acid load by RCCT-28A cells. Mean rates of recovery are indicated by unfilled (presence of K⁺), black (absence of K⁺), and stippled (in the presence of Sch-28080 with K⁺ present) bars. * P<.01 vs. 5 mM K⁺. ** P<.05 vs. 5 mM K⁺, P=NS vs. 0 K⁺.

CCD, in which H,K-ATPase activity was abolished by Sch-28080 under normal conditions, and resistant to Sch-28080 only during K^+ depletion, suggesting up-regulation or mobilization of $HK\alpha_2$ pumps. If basal H,K-ATPase activity in RCCT-28A cells is mediated primarily by $HK\alpha_1$, then post-transcriptional regulation may explain the functional silence of the $HK\alpha_2$ enzymes. However, there is not yet a clear understanding of the Sch-28080 inhibitor profile. In the mammalian expression system (Grishin *et al.*, 1996), the $HK\alpha_2$ activity is inhibited by Sch-28080 at lower concentrations than in the heterologous expression systems. Also, it was not certain that the β isoform paired with the $HK\alpha_2$ subunit in the expression systems is the one that associates with it *in vivo*. Sch-28080 may have an artificially reduced potency in expression systems due to the lack of association of the correct β subunit or some other indirect effect, therefore conclusions reached based on the inhibitor profile must be interpreted cautiously.

$HK\alpha_2$ is sensitive to the inhibitor ouabain (Modyanov *et al.*, 1995; Codina *et al.* 1996; Coughnon *et al.*, 1996; Grishin *et al.*, 1996), while $HK\alpha_1$ has no apparent sensitivity, suggesting a potential means to discriminate on a pharmacological basis between the activity of the two isoforms. However, the primary action of ouabain is inhibition of Na,K-ATPase, so application of ouabain would disturb the Na^+ and K^+ equilibria of the cell. Indeed, in preliminary experiments discussed above, H,K-ATPase activity was observed to be profoundly affected by the presence or absence of Na^+ . Therefore, any effect of ouabain on apparent activities of the α_2 subunit H,K-ATPases could not be distinguished from a secondary effect due to inhibition of Na,K-ATPase. Only when a

great deal more is known about the effect of specific inhibitors on H,K-ATPase isoforms, including any species differences in their potency, would inhibitor studies be valuable.

Fluorescent measurements of pH, recovery rates attributable to H,K-ATPase activity in microperfused CCD in previous studies (Constantinescu *et al.*, 1997; Silver *et al.*, 1993; Silver *et al.*, 1996; Weiner and Milton, 1996; Milton and Weiner, 1997; Silver *et al.*, 1997) have yielded values between 0.03 ± 0.01 and 0.129 ± 0.045 pH U/min. These values are greater than those in the present study, but transport activity in continuous cell lines is typically less robust than that of microperfused tubules. Another continuous cell line of renal origin, a mouse inner medullary collecting duct cell line, has been studied using these techniques and recovery rates of 0.055 ± 0.009 were observed Ono *et al.*, (1996). Species difference and axial heterogeneity of the collecting duct may explain the difference in rates between the two renal cell lines.

A major goal of this project was to define which cell type or types in the kidney contain H,K-ATPase. The RCCT-28A cell line, which has many characteristics of the collecting duct acid-secreting intercalated cell, clearly expresses several H,K-ATPases. Although mRNA and protein level were low, there was detectable H,K-ATPase activity in these cells. Multiple H,K-ATPase isoforms may contribute to this activity.

Understanding of H⁺ and K⁺ transport in the collecting duct by H,K-ATPases must involve investigations into the kinetics and regulation of each isoform. Each H,K-ATPase isoform might have been limited to a different single cell type rather than a single cell containing multiple H,K-ATPases. It was important to determine which of these cases actually occurs. An interesting aspect to these studies is the implication that regulatory processes of the various H,K-ATPase isoforms reside in a single cell type. This would

allow an interplay at the intracellular level between isoforms, perhaps by crosstalk between signal transduction pathways, or perhaps even by physical interaction between different H,K-ATPase subunit isoforms.

PERSPECTIVE AND FUTURE DIRECTIONS

Multiplicity of H,K-ATPase Isoforms in the Kidney

Just in the last decade it has become known that H,K-ATPase is one of the enzymes that contributes to regulation of acid/base balance and potassium homeostasis by the kidney (Doucet and Marsy, 1987; Wingo, 1989). Only recently have the molecules that mediate this activity in the kidney been identified (Modyanov *et al.*, 1991; Callaghan *et al.*, 1995; Ahn and Kone, 1995; Kone and Higham, 1998). This year, evidence was provided by two independent investigations that the NaK β_1 subunit also is a participant in H,K-ATPase activity (DuBose *et al.*, 1998; Kraut *et al.*, 1998). These studies showed that NaK β_1 subunit was the long-sought β subunit that associates with the H,K-ATPase catalytic subunit discovered in distal colon by Crowson and Shull (1992). Thus, there is a great deal of complexity at the molecular level behind the H,K-ATPase activity that was observed in the kidney.

The studies described in this dissertation address the molecular nature of H,K-ATPases in the kidney. A novel HK β subunit variant mRNA (HK β') specific to renal medulla was found by northern analysis. In addition to the canonical transcript, the novel mRNA was expressed in the renal medulla, but not in renal cortex or stomach. The HK β' subunit mRNA may have been a product of medulla-specific alternative splicing at the 5' end of the HK β gene. There was individual variation in rabbits with respect to

relative abundance of HK β and HK β' subunit mRNA in medulla of the kidney, but HK β' was apparently not regulated by dietary K⁺ restriction. The existence of HK β' shows that the nature of H,K-ATPase β subunits, as well as α subunits, is complex and must be considered in understanding H,K-ATPase function in the kidney.

A number of H,K-ATPase α subunit cDNAs have been cloned and sequenced from various species. They might be initially characterized by their tissue distribution. First, there was the well-known gastric isoform HK α_1 subunit cloned from rat (Shull and Lingrel, 1986), pig (Maeda *et al.*, 1988), human (Maeda *et al.*, 1990), rabbit (Bamberg *et al.*, 1992), frog (Mathews *et al.*, 1995), and mouse (Mathews *et al.*, 1995). Second, there were H,K-ATPase catalytic isoforms cloned from human axillary skin (Modyanov *et al.*, 1991), rat distal colon (Crowson and Shull, 1992), guinea pig distal colon (Watanabe *et al.*, 1993), and a partial cDNA from rabbit kidney (Fejes-Toth *et al.* 1995). These were not detected in stomach, but were expressed in numerous other tissues, primarily distal colon. Thus far, at most two P-type H,K-ATPase α subunit isoforms have been identified in any one species. Alternatively spliced variants of the non-gastric isoforms have been found in rat (Kone and Higham, 1998) and rabbit (Campbell *et al.*, 1998).

An H,K-ATPase catalytic subunit has been cloned from the bladder of the toad *Bufo marinus* that had a tissue distribution that contrasted with that seen in mammals (Jaisser *et al.*, 1993). While northern analysis detected the toad HK α transcripts in bladder, it was not observed in either stomach or distal colon. This may reflect differences in physiology between the toad and mammalian species. For example, the toad may not require distal

colonic K^+ reabsorption for K^+ homeostasis. On the other hand, the toad $HK\alpha$ may be the first evidence of an H,K-ATPase subunit that has escaped detection in mammals.

Controversy persists over the nomenclature of H,K-ATPase subunits. Some investigators classify the rat non-gastric $HK\alpha$ as a different H,K-ATPase than the human non-gastric $HK\alpha$. This is based on qualitative differences in inhibitor profile observed for rat and human non-gastric isoforms in heterologous expression systems. Under their nomenclature, rat and human H,K-ATPase subunits are referred to as $HK\alpha_2$ and $HK\alpha_4$, respectively. Quantitative differences in sensitivities to inhibitors may not be a valid measure by which to classify orthologous groups. The Na,K-ATPases also have widely differing qualitative differences between species in their response to inhibitors. As an example ouabain, the widely used "specific" inhibitor of Na,K-ATPases, is highly effective against the pumps in most species other than rat. For rat $NaK\alpha_1$, ouabain is effective only at higher concentrations, but the rat $NaK\alpha_1$ is not thought of as a different $NaK\alpha_1$ than other species. To classify H,K-ATPase isoforms, it might be more valuable to first look at tissue distribution. This would lead to three classes of H,K-ATPase catalytic subunits. $HK\alpha_1$ would continue to denote the gastric H,K-ATPases catalytic subunits, $HK\alpha_2$ would refer to the non-gastric isoforms of H,K-ATPase α subunits, and $HK\alpha_3$ would denote the toad bladder isoform. It became one of the goals of this dissertation to determine how the rabbit $HK\alpha$ isoform related to other H,K-ATPases. Rabbit, rat, and human non-gastric H,K-ATPase cDNA sequences will best fit into a single group based on their tissue distribution and on their phylogenetic relationship.

A search for novel P-type H,K-ATPases expressed in the kidney was performed. The purpose of this was twofold. First, a rabbit HK α_2 sequence was needed to design reagents for study of H,K-ATPase regulation and function in the kidney. In addition, the sequence would be useful to compare and contrast with HK α_2 sequences found in other species to examine their relationship and find important conserved motifs. Second, it was hoped that such a search would find any previously unknown H,K-ATPase catalytic subunit isoforms. The search as conducted was by no means exhaustive, once an interesting fragment of sequence was found, no further attempts were made to find others. However, other investigators also looking for novel H,K-ATPases in rabbit kidney also found the same isoform as described in this work (Fejes-Toth *et al.*, 1995; Jaisser, personal communication). Because of the numerous attempts to expand the number of H,K-ATPases known in human and rat (Shull and Lingrel, 1987; Sverdlov *et al.*, 1987; Modyanov *et al.*, 1991; Crowson and Shull, 1992), it must now be regarded as less likely that completely novel mammalian isoforms will be found.

An HK α subunit isoform was found that had high homology to human and rat HK α_2 . The relationship of the rabbit HK α subunit isoform to other H,K-ATPases and Na,K-ATPases was computationally analyzed by the distance and maximum parsimony algorithms. It was found that sequences for rat and human HK α_2 subunit isoforms were more closely related than HK α_1 or the Na,K-ATPases. The HK α_3 subunit sequence of *Bufo marinus* was slightly more homologous to the HK α_2 subunit sequences than to the HK α_1 sequences. Without more H,K-ATPase sequences related to HK α_3 , its exact relationship to other H,K-ATPases will be difficult to judge.

Kone and Higham (1998) had found an alternatively spliced 5' end of an mRNA that they designated HK α_{2b} . In the work described here, there were two 5' ends found in rabbit as well. The translation start sites of the rabbit and rat alternatively spliced variants are not conserved. In the rat HK α_{2b} mRNA, the start codon is downstream of the splice site (Kone and Higham, 1998), and in the rabbit HK α_{2c} the start codon is upstream of the splice site. For this reason, the rabbit H,K-ATPase catalytic subunit that is the product of alternative splicing was designated HK α_{2c} . However, the pattern of exons and introns at the 5' end of the HK α_2 gene in rat and rabbit was determined to be the same. The relationship shown by phlogenetic analysis and the similarity of alternative splicing implied that rat and rabbit HK α_2 do indeed represent the same gene in different species. An alternatively spliced transcript has not so far been observed for human HK α_2 , but that does not constitute absolute evidence that a similar variant was not transcriptionally competent in human. The human HK α_2 gene sequence (Sverdlov *et al.*, 1996) predicts a translational start codon that was different from both rat and rabbit, downstream of the splice site, but upstream of the first in-frame ATG known for rat. Based on phylogeny, the human HK α_2 was also an ortholog, with its alternatively spliced transcript thus far unseen.

Because of the lack of conservation of splice sites between rat and rabbit alternatively spliced HK α_2 , it was necessary to determine that the start codon in rabbit that gave rise to the longest open reading frame was indeed used. Western analyses using the anti-HK α_2 common and anti-HK α_{2c} antibodies showed a pattern of reactivity consistent with the HK α_{2c} protein being larger than the HK α_{2a} protein. Therefore, the predicted start codon is the one used in rabbit.

The work involved in this dissertation did not succeed in answering the question of why so many H,K-ATPase isoforms exist. With respect to HK α_{2c} , regulation by covalent modification is rendered a less attractive possibility by the lack of conservation of the amino terminal end. The potential phosphorylation sites that were identified by computer analysis in the rabbit are within the amino terminal region that is missing in the rat. Immunohistochemistry showed that the localization of HK α_{2c} within the kidney did not differ widely from that previously found for HK α_1 . HK α_{2c} is apparently not the molecule that is responsible for the basolateral H,K-ATPase activity characterized in proximal tubule and thick ascending limb of Henle by Younes-Ibrahim *et al.* (1995).

The existence of an alternatively spliced isoform offers the possibility of more complex transcriptional regulation. One alternatively spliced isoform may be responsive to different stimuli than another. Perhaps the protein arising from the alternative splicing has different properties, such as kinetic parameters, degradation rates, or internalization rates. Protein interactions have been seen to have major effects on such catalytic properties as ATP binding affinity (Morii *et al.*, 1996), and the amino terminal extension could easily play a role in intra-molecular interaction with other H,K-ATPase molecules or with other proteins. Regulatory proteins could bind the extension to activate or inactivate the enzyme. There is also a possible role of the upstream open reading frames in reducing the efficiency of translation. All these possibilities deserve attention, and work is under way in the Cain laboratory to explore the ways in which HK α_{2a} and HK α_{2c} differ functionally in an expression system.

Cell Type Specificity of H,K-ATPase in the Kidney

H,K-ATPase activity is found at highest level in collecting duct, with some investigators reporting activity in thick ascending limb (Younes-Ibrahim *et al.*, 1995). *In situ* hybridization studies have been conducted that show the distribution of HK α_1 (Ahn and Kone, 1995) and HK β (Campbell-Thompson *et al.*, 1995). Both HK α_1 and HK β share a similar distribution, primarily in collecting duct intercalated cells. A similar cellular distribution was obtained by immunohistochemistry for HK α_1 , with apical polar distribution (Wingo *et al.*, 1990; Haragsim and Bastani, 1996). In immunohistochemical studies using the anti-HK α_{2c} carried out by Dr. Jill Verlander, a similar localization of immunoreactivity was seen, indicating that HK α_{2c} was expressed in generally the same cell types and polarity as HK α_1 and HK β . The implication was that HK α_{2c} represents an H,K-ATPase that has the same qualitative function as other H,K-ATPases, but may have some regulatory differences. The observation that HK α_1 , HK α_{2a} , HK α_{2c} , and HK β were all detected in an acid-secreting intercalated cell line of the cortical collecting duct adds to the weight of the evidence that one of the cell types *in vivo* expressing H,K-ATPase subunits were A-type intercalated cells. It was highly interesting that all these acid pumps, like the V-type H-ATPase (Haragsim and Bastani, 1996) are expressed in the same cells. These cells are uniquely differentiated to mediate acid secretion. Intercalated cells undergo hypertrophy and hyperplasia in animals facing an acid compromise. Because of the capability of that single cell type to increase the overall capability of the kidney for handling an acid state, it is logical that the molecules that participate in acid extrusion may be found in that cell type.

Future Studies

The studies detailed in this dissertation naturally lead to many questions and must leave many of these questions unanswered. The most pressing question upon finding a novel enzyme or variation on an enzyme is what is the significance of the novelty? In the cases of $\text{HK}\beta^+$ and $\text{HK}\alpha_{2c}$, efforts were begun to answer this question, but the answer lies elsewhere in experiments yet to be done. Dietary K^+ restriction was tested as a possible regulatory factor, and mineralocorticoid levels and acid/base status are likely candidates that remain to be examined. Immunohistochemistry failed to reveal any large departure of $\text{HK}\alpha_{2c}$ localization compared to other H,K-ATPases in the kidney, but this localization could change upon disturbances in acid/base, K^+ , or mineralocorticoid status. There are no immunohistochemical studies available for any of the H,K-ATPases under these three conditions.

One would expect that if there were a third mammalian H,K-ATPase α subunit, with the number of investigators searching, that it would have been found. Perhaps there is some sequence-specific difficulty in its amplification or cloning. A difference in size or secondary RNA structure could affect the efficiency with which a novel sequence was amplified. Perhaps RT-PCR reactions using degenerate primers ought to be attempted in greater numbers to increase the chance of finding a truly novel H,K-ATPase, one that is either difficult to process or at a much lower level than other P-type ATPases. The experiment would have to be done many more times. This is practical given current prices for reagents and sequencing. The $\text{HK}\alpha_3$ subunit mRNA fragment was found in the third

reaction; if a novel sequence were present at tenfold less copies per reaction or ten-fold lower efficiency, it should be found in thirty reactions.

There is evidence of an H,K-ATPase in thick ascending limb having different properties than known H,K-ATPases (Younes-Ibrahim *et al.*, 1995; Buffin-Meyer *et al.*, 1997). This is tantalizing evidence that more H,K-ATPase molecules may exist. RT-PCR is now being carried out in glass capillaries with vanishingly small amounts of starting material. Perhaps this could help by allowing individual regions of nephron and collecting duct to be tried as sources for novel H,K-ATPases.

To look at the question of novel H,K-ATPases a different way, it will over the next five to ten years be a simpler task to put an upper limit on how many P-type H,K-ATPases there are. If this number were raised, it would lend weight to the argument for more attempts at finding novel H,K-ATPase molecules. All of the P-type H,K-ATPases pointed to by earlier work by Shull and Lingrel (1987) and Sverdlov *et al.* (1987) have now been associated with a specific enzyme. Southern analysis of restriction digested genomic DNA probed at low stringency by probes corresponding to well-conserved regions of P-type ATPases could help determine the number of P-type Na,K- and H,K-ATPases exist. With the sequencing of the human genome, such searches will be able to be carried out *in silico*. This will allow many more parameters in the experiment.

Alternative splicing has now been observed in rat and rabbit HK α_2 . It would be quite interesting to try to observe alternative splicing of a transcript in human HK α_2 as well. A positive result would constitute one further piece of evidence that the rat, rabbit, and human genes are analogs of the same gene in different species. Another piece of interesting evidence could come from chromosomal location of these genes. If the genes

were found in syntenic regions, it would strengthen the argument for their being analogs of the same gene in different species considerably.

Much work remains to be done in appreciating the localization of the H,K-ATPases in the kidney. Double-labeling immunohistochemistry using well-characterized markers would help define the cell types in which each H,K-ATPase subunit isoform was expressed. Quality antibodies are available for carbonic anhydrase, a marker for intercalated cells, and for band 3 protein, a marker for A-type intercalated cells. This would help refine our knowledge of the cell types in which H,K-ATPase is expressed. At present, an experienced eye is the main tool for determining cell types. With double-labelling experiments, the conclusions would be more concrete.

A major advance in making H,K-ATPase activity measurements would be to find inhibitors that worked at low concentrations with high specificity. Characterization has been limited to the effects of Sch-28080 and ouabain action on $\text{HK}\alpha_2$, but literature is conflicting with respect to the concentrations observed for inhibition constants (Modyanov *et al.*, 1995; Codina *et al.*, 1996; Grishin *et al.*, 1996; Cougnon *et al.*, 1996). These conflicts need to be resolved, and other H,K-ATPase inhibitors, such as omeprazole or A80915A, might be tried to measure their action on the $\text{HK}\alpha_2$ species. These experiments would preferably be conducted in mammalian expression systems. Members of the Cain laboratory are in the process of developing such a system. With useful information about inhibitor profiles, studies in perfused tubules might be performed to determine the cell types that have the various H,K-ATPase activities. Protocols for determining cell types functionally in perfused tubules are well known.

To answer broader questions, such as how does the kidney respond to acid/base disturbances, K^+ restriction, or mineralocorticoid levels, new techniques will be useful. There are now blot arrays available that have many cDNAs attached. These may be probed with labelled mRNA from controlled and treated animals and differentials in many mRNA levels observed in parallel. Each cDNA had an assigned location on the blot, so one can quickly find what transcript is being regulated. For a more all-encompassing approach to these experiments, microarrays may be used that can have even more cDNAs on them. These methods study many regulatory processes in parallel, and could be a quick way to pinpoint interesting responses. However, these techniques require first that the responsive element was identified and sequenced. That was the goal of this work, to identify and characterize H,K-ATPase molecules, elements that play a role in the biological processes carried out in the kidney.

To that end, variants of HK β and HK α_2 have been found and their sequences determined. Such tools as cDNA probes and antibodies have been produced. These are a prerequisite for determining the significance of the novel H,K-ATPases. It was shown that HK α_{2c} protein is expressed and that the predicted start codon is used. Evidence by immunohistochemistry and the presence of mRNA and protein in an A-type intercalated collecting duct cell line showed that acid-secreting intercalated cells are a major cell type in which HK α_{2c} pumps may be found. The HK α_{2c} pump adds to the complement of transporters that may mediate acid secretion and K^+ reabsorption in those cells. These results define H,K-ATPase varieties that were not known when this work began.

REFERENCES

- Addison, W. L. T. The use of sodium chloride, potassium chloride, sodium bromide, and potassium bromide in cases of arterial hypertension which are amenable to potassium chloride. *Can Med Assoc H* 18: 281-5, 1928.
- Ahn, K. Y., and B. C. Kone. Expression and cellular localization of mRNA encoding the "gastric" isoform of H(+)-K(+)-ATPase alpha-subunit in rat kidney. *Am J Physiol* 268: F99-109, 1995.
- Ahn, K. Y., K. Y. Park, K. K. Kim, and B. C. Kone. Chronic hypokalemia enhances expression of the H(+)-K(+)-ATPase alpha 2-subunit gene in renal medulla. *Am J Physiol* 271: F314-21, 1996.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. Basic local alignment search tool. *J Mol Biol* 215: 403-10, 1990.
- Ambard, L., and C. Beaujard. Causes de l'hypertension artérielle. *Arch Gen Med* 10: 520-33, 1904.
- Arend, L. J., J. S. Handler, J. S. Rhim, F. Gusovsky, and W. S. Spielman. Adenosine-sensitive phosphoinositide turnover in a newly established renal cell line. *Am J Physiol* 256: F1067-74, 1989.
- Bairoch, A., and R. Apweiler. The SWISS-PROT protein sequence data bank and its new supplement TREMBL. *Nucleic Acids Res* 24: 21-5, 1996.
- Bamberg, K., F. Mercier, M. A. Reuben, Y. Kobayashi, K. B. Munson, and G. Sachs. cDNA cloning and membrane topology of the rabbit gastric H+/K(+)-ATPase alpha-subunit. *Biochim Biophys Acta* 1131: 69-77, 1992.
- Bamberg, K., and G. Sachs. Topological analysis of H+,K(+)-ATPase using in vitro translation. *J Biol Chem* 269: 16909-19, 1994.
- Bastani, B. Colocalization of H-ATPase and H,K-ATPase immunoreactivity in the rat kidney. *J Am Soc Nephrol* 5: 1476-82, 1995.
- Bello Reuss, E. Characterization of acid-base transport mechanisms in the kidney cell line RCCT-28A. *Kidney Int* 43: 173-81, 1993.

Besancon, M., J. M. Shin, F. Mercier, K. Munson, M. Miller, S. Hersey, and G. Sachs. Membrane topology and omeprazole labeling of the gastric H^+ , K^+ -adenosinetriphosphatase. *Biochemistry* 32: 2345-55, 1993.

Blanco, G., J. C. Koster, and R. W. Mercer. The alpha subunit of the Na , K -ATPase specifically and stably associates into oligomers. *Proc Natl Acad Sci USA* 91: 8542-6, 1994.

Buffin Meyer, B., M. Younes Ibrahim, C. Barlet Bas, L. Cheval, S. Marsy, and A. Doucet. K depletion modifies the properties of Sch-28080-sensitive K -ATPase in rat collecting duct. *Am J Physiol* 272: F124-31, 1997.

Burnatowska-Hledin, M. A., and W. S. Spielman. Vasopressin increases cytosolic free calcium in LLC-PK1 cells through a V_1 -receptor. *Am J Physiol* 253: F328-32, 1987.

Callaghan, J. M., S. S. Tan, M. A. Khan, K. A. Curran, W. G. Campbell, A. J. Smolka, B. H. Toh, P. A. Gleeson, C. S. Wingo, B. D. Cain, and et al. Renal expression of the gene encoding the gastric H^+ - K^+ -ATPase beta-subunit. *Am J Physiol* 268: F363-74, 1995.

Campbell Thompson, M. L., J. W. Verlander, K. A. Curran, W. G. Campbell, B. D. Cain, C. S. Wingo, and J. E. McGuigan. In situ hybridization of H - K -ATPase beta-subunit mRNA in rat and rabbit kidney. *Am J Physiol* 269: F345-54, 1995.

Canfield, V. A., and R. Levenson. Structural organization and transcription of the mouse gastric H^+ , K^+ -ATPase beta subunit gene. *Proc Natl Acad Sci USA* 88: 8247-51, 1991.

Cheval, L., C. Barlet Bas, C. Khadouri, E. Feraille, S. Marsy, and A. Doucet. K^+ -ATPase-mediated Rb^+ transport in rat collecting tubule: modulation during K^+ deprivation. *Am J Physiol* 260: F800-5, 1991.

Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-9, 1987.

Chow, D. C., C. M. Browning, and J. G. Forte. Gastric H^+ - K^+ -ATPase activity is inhibited by reduction of disulfide bonds in beta-subunit. *Am J Physiol* 263: C39-46, 1992.

Chow, D. C., and J. G. Forte. Characterization of the beta-subunit of the H^+ - K^+ -ATPase using an inhibitory monoclonal antibody. *Am J Physiol* 265: C1562-70, 1993.

Codina, J., J. T. Delmas-Mata, and D. T. D. Jr. The alpha-subunit of the colonic H⁺,K⁺-ATPase assembles with the beta 1-Na⁺,K⁺-ATPase in kidney and distal colon. *J Biol Chem* 273: 7894-9, 1998.

Codina, J., B. C. Kone, J. T. Delmas Mata, and T. D. DuBose, Jr. Functional expression of the colonic H⁺,K⁺-ATPase alpha-subunit. Pharmacologic properties and assembly with X⁺,K⁺-ATPase beta-subunits. *J Biol Chem* 271: 29759-63, 1996.

Colonna, T. E., L. Huynh, and D. M. Fambrough. Subunit interactions in the Na₂K-ATPase explored with the yeast two-hybrid system. *J Biol Chem* 272: 12366-72, 1997.

Constantinescu, A., R. B. Silver, and L. M. Satlin. H-K-ATPase activity in PNA-binding intercalated cells of newborn rabbit cortical collecting duct. *Am J Physiol* 272: F167-77, 1997.

Cougnon, M., G. Planelles, M. S. Crowson, G. E. Shull, B. C. Rossier, and F. Jaisser. The rat distal colon P-ATPase alpha subunit encodes a ouabain-sensitive H⁺, K⁺-ATPase. *J Biol Chem* 271: 7277-80, 1996.

Courtois-Coutry, N., D. Roush, V. Rajendran, J. B. McCarthy, J. Geibel, M. Kashgarian, and M. J. Caplan. A tyrosine-based signal targets H/K-ATPase to a regulated compartment and is required for the cessation of gastric acid secretion. *Cell* 90: 501-10, 1997.

Cox, K. H., and J. V. Cox. Variant chicken AE1 anion exchangers possess divergent NH(2)-terminal cytoplasmic domains. *Am J Physiol* 268: F503-13, 1995.

Crowson, M. S., and G. E. Shull. Isolation and characterization of a cDNA encoding the putative distal colon H⁺,K⁽⁺⁾-ATPase. Similarity of deduced amino acid sequence to gastric H⁺,K⁽⁺⁾-ATPase and Na⁺,K⁽⁺⁾-ATPase and mRNA expression in distal colon, kidney, and uterus. *J Biol Chem* 267: 13740-8, 1992.

Curran, K. A., M. J. Hebert, B. D. Cain, and C. S. Wingo. Evidence for the presence of a K-dependent acidifying adenosine triphosphatase in the rabbit renal medulla. *Kidney Int* 42: 1093-8, 1992.

Dafnis, E., M. Spohn, B. Lonis, N. A. Kurtzman, and S. Sabatini. Vanadate causes hypokalemic distal renal tubular acidosis. *Am J Physiol* 262: F449-53, 1992.

Davis, L. G., W. M. Kuehl, and J. F. Battey. *Basic Methods in Molecular Biology*, 2nd edition. Norwalk, CT: Appleton and Lange, 1994.

Dietl, P., N. Kizer, and B. A. Stanton. Conductive properties of a rabbit cortical collecting duct cell line: regulation by isoproterenol. *Am J Physiol* 262: F578-82, 1992.

Dietl, P., and B. A. Stanton. Chloride channels in apical and basolateral membranes of CCD cells (RCCT-28A) in culture. *Am J Physiol* 263: F243-50, 1992.

Doucet, A., and S. Marsy. Characterization of K-ATPase activity in distal nephron: stimulation by potassium depletion. *Am J Physiol* 253: F418-23, 1987.

DuBose, T. D., Jr., J. Codina, A. Burges, and T. A. Pressley. Regulation of H(+)-K(+)-ATPase expression in kidney. *Am J Physiol* 269: F500-7, 1995.

Farley, R. A., and L. D. Faller. The amino acid sequence of an active site peptide from the H,K-ATPase of gastric mucosa. *J Biol Chem* 260: 3899-901, 1985.

Fejes-Toth, G., E. Rusvai, K. A. Longo, and A. Naray Fejes-Toth. Expression of colonic H-K-ATPase mRNA in cortical collecting duct: Regulation by acid/base balance. *Am J Physiol* 269: F551-7, 1995.

Frohman, M. A., M. K. Dush, and G. R. Martin. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* 85: 8998-9002, 1988.

Ganser, A. L., and J. G. Forte. K⁺-stimulated ATPase in purified microsomes of bullfrog oxyntic cells. *Biochim Biophys Acta* 307: 169-80, 1973.

Garg, L. C. Respective roles of H-ATPase and H-K-ATPase in ion transport in the kidney. *J Am Soc Nephrol* 2: 949-60, 1991.

Garg, L. C., and N. Narang. Ouabain-insensitive K-adenosine triphosphatase in distal nephron segments of the rabbit. *J Clin Invest* 81: 1204-8, 1988.

Garg, L. C., and N. Narang. Suppression of ouabain-insensitive K-ATPase activity in rabbit nephron segments during chronic hyperkalemia. *Ren Physiol Biochem* 12: 295-301, 1989.

Geering, K., I. Theulaz, F. Verrey, M. T. Hauptle, and B. C. Rossier. A role for the beta-subunit in the expression of functional Na⁺-K⁺-ATPase in *Xenopus* oocytes. *Am J Physiol* 257: C851-8, 1989.

Grishin, A. V., M. O. Bevensee, N. N. Modyanov, V. Rajendran, W. F. Boron, and M. J. Caplan. Functional expression of the cDNA encoded by the human ATP1A1 gene. *Am J Physiol* 271: F539-51, 1996.

Grishin, A. V., V. E. Sverdlov, M. B. Kostina, and N. N. Modyanov. Cloning and characterization of the entire cDNA encoded by ATP1A1--a member of the human Na,K/H,K-ATPase gene family. *FEBS Lett* 349: 144-50, 1994.

Guntupalli, J., M. Onuigbo, S. Wall, R. J. Alpern, and T. D. DuBose, Jr. Adaptation to low-K⁺ media increases H⁺-K⁺-ATPase but not H⁺-ATPase-mediated pHi recovery in OMCD1 cells. *Am J Physiol* 273: C558-71, 1997.

Gustin, M. C., and D. B. Goodman. Isolation of brush-border membrane from the rabbit descending colon epithelium. Partial characterization of a unique K⁺-activated ATPase. *J Biol Chem* 256: 10651-6, 1981.

Guyton, A. C., and J. E. Hall. *Textbook of Medical Physiology*. Philadelphia, PA: W. B. Saunders, 1996.

Hall, K., G. Perez, G. Sachs, and E. Rabon. Identification of H⁺/K⁺-ATPase alpha,beta-heterodimers. *Biochim Biophys Acta* 1077: 173-9, 1991.

Hays, S. R., and R. J. Alpern. Inhibition of Na⁺-independent H⁺ pump by Na⁺-induced changes in cell Ca²⁺. *J Gen Physiol* 98: 791-813, 1991.

Hebert, H., Y. Xian, I. Hacksell, and S. Mardh. Two-dimensional crystals of membrane-bound gastric H,K-ATPase. *FEBS Lett* 299: 159-62, 1992.

Hersey, S. J., and G. Sachs. Gastric acid secretion. *Physiol Rev* 75: 155-89, 1995.

Herzlinger, D. A., T. G. Easton, and G. K. Ojakian. The MDCK epithelial cell line expresses a cell surface antigen of the kidney distal tubule. *J Cell Biol* 93: 269-77, 1982.

Horisberger, J. D., P. Jaunin, M. A. Reuben, L. S. Lasater, D. C. Chow, J. G. Forte, G. Sachs, B. C. Rossier, and K. Geering. The H,K-ATPase beta-subunit can act as a surrogate for the beta-subunit of Na,K-pumps. *J Biol Chem* 266: 19131-4, 1991.

INTERSALT. Intersalt: an international study of electrolyte excretion and blood pressure. Results for 24 hour urinary sodium and potassium excretion. Intersalt Cooperative Research Group. *BMJ* 297: 319-28, 1988.

Jaissier, F., B. Escoubet, N. Coutry, E. Eugene, J. P. Bonvalet, and N. Farman. Differential regulation of putative K⁺-ATPase by low-K⁺ diet and corticosteroids in rat distal colon and kidney. *Am J Physiol* 270: C679-87, 1996.

Jaissier, F., J. D. Horisberger, K. Geering, and B. C. Rossier. Mechanisms of urinary K⁺ and H⁺ excretion: primary structure and functional expression of a novel H,K-ATPase. *J Cell Biol* 123: 1421-9, 1993.

Jameson, B. A., and H. Wolf. The antigenic index: a novel algorithm for predicting antigenic determinants. *Comput Appl Biosci* 4: 181-6, 1988.

Kone, B. C. Structural diversity of the colonic H(+)-K(+)-ATPase a subunit (HKalpha2) gene: Cloning and intrarenal distribution of alternative forms. *J Am Soc Nephrol* 7: 1283, 1996.

Kone, B. C., and S. C. Higham. A novel N-terminal splice variant of the rat H+-K+-ATPase alpha2 subunit. Cloning, functional expression, and renal adaptive response to chronic hypokalemia. *J Biol Chem* 273: 2543-52, 1998.

Koster, J. C., G. Blanco, and R. W. Mercer. A cytoplasmic region of the Na,K-ATPase alpha-subunit is necessary for specific alpha/alpha association. *J Biol Chem* 270: 14332-9, 1995.

Kraut, J. A., J. Hiura, M. Besancon, A. Smolka, G. Sachs, and D. Scott. Effect of hypokalemia on the abundance of HKalpha1 and HKalpha2 protein in the rat kidney. *Am J Physiol* 272: F744-50, 1997.

Kraut, J. A., J. Hiura, J. M. Shin, S. A., S. G., and S. D. The Na(+)-K(+)-ATPase beta 1 subunit is associated with the HK alpha 2 protein in the rat kidney. *Kidney Int* 53: 958-62, 1998.

Kraut, J. A., F. Starr, G. Sachs, and M. Reuben. Expression of gastric and colonic H(+)-K(+)-ATPase in the rat kidney. *Am J Physiol* 268: F581-7, 1995.

Lee, J., G. Simpson P. Scholes. An ATPase from dog gastric mucosa: changes of outer pH in suspensions of membrane vesicles accompanying ATP hydrolysis. *Biochem Biophys Res Commun* 60: 825-32, 1974

Lee, J., V. M. Rajendran, A. S. Mann, M. Kashgarian, and H. J. Binder. Functional expression and segmental localization of rat colonic K-adenosine triphosphatase. *J Clin Invest* 96: 2002-8, 1995.

Lifton, R. P. Molecular genetics of human blood pressure variation. *Science* 272: 676-80, 1996.

Maeda, M., J. Ishizaki, and M. Futai. cDNA cloning and sequence determination of pig gastric (H+ + K+)-ATPase. *Biochem Biophys Res Commun* 157: 203-9, 1988.

Maeda, M., K. Oshiman, S. Tamura, and M. Futai. Human gastric (H+ + K+)-ATPase gene. Similarity to (Na+ + K+)-ATPase genes in exon/intron organization but difference in control region. *J Biol Chem* 265: 9027-32, 1990.

Maeda, M., K. Oshiman, S. Tamura, S. Kaya, S. Mahmood, M. A. Reuben, L. S. Lasater, G. Sachs, and M. Futai. The rat H+/K(+)-ATPase beta subunit gene and recognition of its control region by gastric DNA binding protein. *J Biol Chem* 266: 21584-8, 1991.

Marsy, S., J. M. Elalouf, and A. Doucet. Quantitative RT-PCR analysis of mRNAs encoding a colonic putative H, K-ATPase alpha subunit along the rat nephron: effect of K⁺ depletion. *Pflügers Arch* 432: 494-500, 1996.

Mathews, P. M., D. Claeys, F. Jaisser, K. Geering, J. D. Horisberger, J. P. Kraehenbuhl, and B. C. Rossier. Primary structure and functional expression of the mouse and frog alpha-subunit of the gastric H(+)-K(+)-ATPase [published erratum appears in *Am J Physiol* 1995 Oct;269(4 Pt 1):section C following table of contents]. *Am J Physiol* 268: C1207-14, 1995.

Meneton, P., P. J. Schultheis, J. Greeb, M. L. Nieman, L. H. Liu, L. L. Clarke, J. J. Duffy, T. Doetschman, J. N. Lorenz, and G. E. Shull. Increased sensitivity to K⁺ deprivation in colonic H,K-ATPase-deficient mice. *J Clin Invest* 101: 536-42, 1998.

Mercier, F., D. Bayle, M. Besancon, T. Joys, J. M. Shin, M. J. Lewin, C. Prinz, M. A. Reuben, A. Soumarmon, H. Wong, and et al. Antibody epitope mapping of the gastric H⁺/K⁺-ATPase. *Biochim Biophys Acta* 1149: 151-65, 1993.

Merck, S., and Dohme Research Laboratories. *The Merck Manual of Diagnosis and Therapy*. Rahway, NJ: Merck, Sharpe, and Dohme Laboratories, 1992.

Mills, J. W., E. M. Schwiebert, and B. A. Stanton. Evidence for the role of actin filaments in regulating cell swelling. *J Exp Zool* 268: 111-20, 1994.

Milton, A. E., and I. D. Weiner. Intracellular pH regulation in the rabbit cortical collecting duct A-type intercalated cell. *Am J Physiol* 273: F340-7, 1997.

Modyanov, N. N., P. M. Mathews, A. V. Grishin, P. Beguin, A. T. Beggah, B. C. Rossier, J. D. Horisberger, and K. Geering. Human ATP1A1 gene encodes a ouabain-sensitive H-K-ATPase. *Am J Physiol* 269: C992-7, 1995.

Modyanov, N. N., K. E. Petrukhin, V. E. Sverdlov, A. V. Grishin, M. Y. Orlova, M. B. Kostina, O. I. Makarevich, N. E. Broude, G. S. Monastyrskaya, and E. D. Sverdlov. The family of human Na,K-ATPase genes. ATP1A1 gene is transcriptionally competent and probably encodes the related ion transport ATPase. *FEBS Lett* 278: 91-4, 1991.

Morii, M., Y. Hayata, K. Mizoguchi, and N. Takeguchi. Oligomeric regulation of gastric H⁺,K⁺-ATPase. *J Biol Chem* 271: 4068-72, 1996.

Munson, K. B., C. Gutierrez, V. N. Balaji, K. Ramnarayan, and G. Sachs. Identification of an extracytoplasmic region of H⁺,K(+)-ATPase labeled by a K(+)-competitive photoaffinity inhibitor. *J Biol Chem* 266: 18976-88, 1991.

Munson, K. B., and G. Sachs. Inactivation of H^+,K^+ -ATPase by a K^+ -competitive photoaffinity inhibitor. *Biochemistry* 27: 3932-8, 1988.

Newman, P. R., and G. E. Shull. Rat gastric H,K -ATPase beta-subunit gene: intron/exon organization, identification of multiple transcription initiation sites, and analysis of the 5'-flanking region. *Genomics* 11: 252-62, 1991.

Nilwarangkar, S., S. Nimmannit, V. Chaovakul, W. Sussaengrat, S. Ong-Aj-Yooth, S. Vasuvattakul, P. Pidetcha, and P. Malasit. Endemic primary distal renal tubular acidosis in Thailand. *Quarterly Journal of Medicine* 74: 289-302, 1990.

Okusa, M. D., R. J. Unwin, H. Velazquez, G. Giebisch, and F. S. Wright. Active potassium absorption by the renal distal tubule. *Am J Physiol* 262: F488-93, 1992.

Ono, S., J. Guntupalli, and T. D. DuBose, Jr. Role of $H(+)-K(+)$ -ATPase in pH_i regulation in inner medullary collecting duct cells in culture. *Am J Physiol* 270: F852-61, 1996.

Oshiman, K., K. Motojima, S. Mahmood, A. Shimada, S. Tamura, M. Maeda, and M. Futai. Control region and gastric specific transcription of the rat $H^+,K(+)$ -ATPase alpha subunit gene. *FEBS Lett* 281: 250-4, 1991.

Pettitt, J. M., D. C. Humphris, S. P. Barrett, B. H. Toh, I. R. van Driel, and P. A. Gleeson. Fast freeze-fixation/freezing-substitution reveals the secretory membranes of the gastric parietal cell as a network of helically coiled tubule. A new model for parietal cell transformation. *J Cell Sci* 108: 1127-41, 1995.

Pfaller, W., G. Gstraunthaler, U. Kersting, and H. Oberleithner. Carbonic anhydrase activity in Madin Darby canine kidney cells. Evidence for intercalated cell properties. *Ren Physiol Biochem* 12: 328-37, 1989.

Rabon, E. C., R. D. Gunther, S. Bassilian, and E. S. Kempner. Radiation inactivation analysis of oligomeric structure of the H,K -ATPase. *J Biol Chem* 263: 16189-94, 1988.

Raussens, V., H. de Jongh, M. Pezolet, J. M. Ruysschaert, and E. Goormaghtigh. Secondary structure of the intact H^+,K^+ -ATPase and of its membrane-embedded region. An attenuated total reflection infrared spectroscopy, circular dichroism and Raman spectroscopy study. *Eur J Biochem* 252: 261-7, 1998.

Reuben, M. A., L. S. Lasater, and G. Sachs. Characterization of a beta subunit of the gastric H^+/K^+ -transporting ATPase. *Proc Natl Acad Sci USA* 87: 6767-71, 1990.

Sachs, G., H. H. Chang, E. Rabon, R. Schackman, M. Lewin, and G. Saccomani. A nonelectrogenic H⁺ pump in plasma membranes of hog stomach. *J Biol Chem* 251: 7690-8, 1976.

Sachs, G., L. D. Faller, and E. Rabon. Proton/hydroxyl transport in gastric and intestinal epithelia. *J Membr Biol* 64: 123-35, 1982.

Sangan, P., V. M. Rajendran, A. S. Mann, M. Kashgarian, and H. J. Binder. Regulation of colonic H-K-ATPase in large intestine and kidney by dietary Na depletion and dietary K depletion. *Am J Physiol* 272: C685-96, 1997.

Sarvazyan, N. A., N. N. Modyanov, and A. Askari. Intersubunit and intrasubunit contact regions of Na⁺/K⁺-ATPase revealed by controlled proteolysis and chemical cross-linking. *J Biol Chem* 270: 26528-32, 1995.

Schwiebert, E. M., K. H. Karlson, P. A. Friedman, P. Dietl, W. S. Spielman, and B. A. Stanton. Adenosine regulates a chloride channel via protein kinase C and a G protein in a rabbit cortical collecting duct cell line. *J Clin Invest* 89: 834-41, 1992.

Schwiebert, E. M., J. W. Mills, and B. A. Stanton. Actin-based cytoskeleton regulates a chloride channel and cell volume in a renal cortical collecting duct cell line. *J Biol Chem* 269: 7081-9, 1994.

Shamraj, O. I., J. B. Lingrel. A putative fourth Na⁺/K⁺-ATPase α -subunit gene is expressed in testis. *Proc Natl Acad Sci USA* 91:12952-6, 1994.

Shin, J. M., M. Besancon, A. Simon, and G. Sachs. The site of action of pantoprazole in the gastric H⁺/K⁺-ATPase. *Biochim Biophys Acta* 1148: 223-33, 1993.

Shin, J. M., and G. Sachs. Identification of a region of the H,K-ATPase alpha subunit associated with the beta subunit. *J Biol Chem* 269: 8642-6, 1994.

Shull, G. E., and J. B. Lingrel. Molecular cloning of the rat stomach (H⁺ + K⁺)-ATPase. *J Biol Chem* 261: 16788-91, 1986.

Shull, M. M., and J. B. Lingrel. Multiple genes encode the human Na⁺/K⁺-ATPase catalytic subunit. *Proc Natl Acad Sci USA* 84: 4039-43, 1987.

Silver, R. B., and G. Frindt. Functional identification of H-K-ATPase in intercalated cells of cortical collecting tubule. *Am J Physiol* 264: F259-66, 1993.

Silver, R. B., G. Frindt, P. Mennitt, and L. M. Satlin. Characterization and regulation of H-K-ATPase in intercalated cells of rabbit cortical collecting duct. *J Exp Zool* 279: 443-55, 1997.

Silver, R. B., P. A. Mennitt, and L. M. Satlin. Stimulation of apical H-K-ATPase in intercalated cells of cortical collecting duct with chronic metabolic acidosis. *Am J Physiol* 270: F539-47, 1996.

Sitprija, V., E.-O. S., R. Suvanapha, P. Kullavanijaya, and S. Chinayon. Gastric hypoacidity in distal renal tubular acidosis. *Nephron* 50: 395-6, 1988.

Smolka, A., L. Alverson, R. Fritz, K. Swiger, and R. Swiger. Gastric H,K-ATPase topography: amino acids 888-907 are cytoplasmic. *Biochem Biophys Res Commun* 180: 1356-64, 1991.

Smolka, A., and K. M. Swiger. Site-directed antibodies as topographical probes of the gastric H,K-ATPase alpha-subunit. *Biochim Biophys Acta* 1108: 75-85, 1992.

Spielman, W. S., W. K. Sonnenburg, M. L. Allen, L. J. Arend, K. Gerozissis, and W. L. Smith. Immunodissection and culture of rabbit cortical collecting tubule cells. *Am J Physiol* 251: F348-57, 1986.

Sverdlov, E. D., G. S. Monastyrskaya, N. E. Broude, A. Ushkaryov Yu, R. L. Allikmets, A. M. Melkov, V. Smirnov Yu, I. V. Malyshev, I. E. Dulobova, K. E. Petrukhin, A. V. Grishin, N. I. Kijatkin, M. B. Kostina, V. E. Sverdlov, N. N. Modyanov, and Y. A. Ovchinnikov. The family of human Na⁺,K⁺-ATPase genes. No less than five genes and/or pseudogenes related to the alpha-subunit. *FEBS Lett* 217: 275-8, 1987.

Sverdlov, V. E., M. B. Kostina, and N. N. Modyanov. Genomic organization of the human ATP1A1 gene encoding a ouabain-sensitive H,K-ATPase. *Genomics* 32: 317-27, 1996.

Tamura, S., K. Oshiman, T. Nishi, M. Mori, M. Maeda, and M. Futai. Sequence motif in control regions of the H⁺/K⁺ ATPase alpha and beta subunit genes recognized by gastric specific nuclear protein(s). *FEBS Lett* 298: 137-41, 1992.

Thomas, J. A., R. N. Buchsbaum, A. Zimniak, and E. Racker. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* 18: 2210-8, 1979.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-80, 1994.

Valentich, J. D. Morphological similarities between the dog kidney cell line MDCK and the mammalian cortical collecting tubule. *Ann N Y Acad Sci* 372: 384-405, 1981.

Van Driel, I. R., and J. M. Callaghan. Proton and potassium transport by H⁺/K⁺-ATPases. *Clin Exp Pharmacol Physiol* 22: 952-60, 1995.

van Driel, I. R., and J. M. Callaghan. Proton and potassium transport by H⁺/K⁺-ATPases. *Clin Exp Pharmacol Physiol* 22: 952-60, 1995.

Walderhaug, M. O., R. L. Post, G. Saccomani, R. T. Leonard, and D. P. Briskin. Structural relatedness of three ion-transport adenosine triphosphatases around their active sites of phosphorylation. *J Biol Chem* 260: 3852-9, 1985.

Weiner, I. D., and L. L. Hamm. Use of fluorescent dye BCECF to measure intracellular pH in cortical collecting tubule. *Am J Physiol* 256: F957-64, 1989.

Weiner, I. D., and A. E. Milton. H⁺(+)-K⁺(-)-ATPase in rabbit cortical collecting duct B-type intercalated cell. *Am J Physiol* 270: F518-30, 1996.

Whelton, P. K. Potassium and Blood Pressure. In: *Hypertension Primer*, edited by J. L. Izzo, Black, H. R. Dallas, TX: American Heart Association, 1993, p. 170-2.

Willardson, B. M., B. J. Thevenin, M. L. Harrison, W. M. Kuster, M. D. Benson, and P. S. Low. Localization of the ankyrin-binding site on erythrocyte membrane protein, band 3. *J Biol Chem* 264: 15893-9, 1989.

Wingo, C. S. Potassium transport by medullary collecting tubule of rabbit: effects of variation in K intake. *Am J Physiol* 253: F1136-41, 1987.

Wingo, C. S. Active proton secretion and potassium absorption in the rabbit outer medullary collecting duct. Functional evidence for proton-potassium-activated adenosine triphosphatase. *J Clin Invest* 84: 361-5, 1989.

Wingo, C. S., and B. D. Cain. The renal H-K-ATPase: physiological significance and role in potassium homeostasis. *Annu Rev Physiol* 55: 323-47, 1993.

Wingo, C. S., K. M. Madsen, A. Smolka, and C. C. Tisher. H-K-ATPase immunoreactivity in cortical and outer medullary collecting duct. *Kidney Int* 38: 98S-90, 1990.

Wingo, C. S., and A. J. Smolka. Function and structure of H-K-ATPase in the kidney [editorial]. *Am J Physiol* 269: F1-16, 1995.

Wolfe, M. M., and A. H. Soll. The physiology of gastric acid secretion. *N Engl J Med* 319: 1707-15, 1988.

Younes Ibrahim, M., C. Barlet Bas, B. Buffin Meyer, L. Cheval, R. Rajerison, and A. Doucet. Ouabain-sensitive and -insensitive K-ATPases in rat nephron: effect of K depletion. *Am J Physiol* 268: F1141-7, 1995.

Zhang, P., C. Toyoshima, K. Yonejura, N. M. Green, and D. L. Stokes. Structure of the calcium pump from sarcoplasmic reticulum at 8-A resolution. *Nature* 392: 835-839, 1998.

BIOGRAPHICAL SKETCH

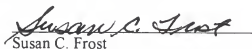
Grady Campbell was born in Clearwater, Florida, on February 1, 1955. He graduated from Charles E. Jordan High School of Durham, North Carolina, in June 1973. The following September he entered Emory University in Atlanta, Georgia, and in June 1977 he received a Bachelor of Science degree, with a major in physics. In August of that year he began employment at EG&G ORTEC in Oak Ridge, Tennessee. In the fall of 1979 he enrolled as a part-time student at the University of Tennessee, Knoxville, and began study toward a master's degree under the direction of Carrol R. Bingham, with a major in physics. His thesis was entitled The Decay of Mass-separated ^{193}Pb to ^{193}Tl ; the degree was awarded in August 1985. In April of the following year he began employment at California Institute of Technology in Pasadena, California. In August 1992 he enrolled as a graduate student in the Department of Biochemistry and Molecular Biology at the University of Florida in Gainesville, Florida. That December, he joined Brian D. Cain's laboratory where he carried out the work described in this dissertation. Upon completing the requirements for his degree, he is joining Frank W. Booth's laboratory at the University of Texas-Houston Medical School in Houston, Texas, where he will find and study genes involved in conferring the benefit of exercise on cardiovascular disease.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Brian D. Cain, Chair
Associate Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Susan C. Frost
Associate Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



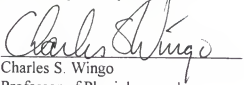
Michael S. Kilberg
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Harry S. Nick
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Charles S. Wingo
Professor of Physiology and
Pharmacology

This disssertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1998



Dean, College of Medicine



Dean, Graduate School